Understanding the Eco-evolutionary Dynamics Underpinning Changes in Air-Liquid Interface Biofilms in Radiating Populations and Multi-Species Communities

A thesis submitted for the degree of Doctor of Philosophy, PhD

by

Robyn Shannon Jerdan

School of Applied Science
Abertay University.

January, 2021

Table of Contents

Declaration

Candidate's declarations:

I, Miss Robyn Shannon Jerdan, hereby certify that this thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy (PhD), Abertay University, is wholly my own work unless otherwise referenced or acknowledged. This work has not been submitted for any other qualification at any other academic institution.

Signed 

Date……15/01/2021……..

Supervisor's declaration:

I, Dr Andrew Spiers hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy (PhD), Abertay University in Abertay University and that the candidate is qualified to submit this thesis in application for that degree.

Signed 

Date……15/01/2021……

Certificate of Approval

I certify that this is a true and accurate version of the thesis approved by the examiners, and that all relevant ordinance regulations have been fulfilled.

Supervisor 

Date……15/01/2021……..

Acknowledgemnts

I would firstly like to thank my principal supervisor Dr Andrew Spiers. For believing and pushing me to exceed my potential, your knowledge and expertise has been invaluable throughout my PhD and I would not have achieved so much over the last three years without your support. I truly hope we can work together again in the future. I thank my secondary supervisor Dr Scott Cameron for your input in enhancing publications and my thesis, and your guidance as a mentor to help me achieve associate fellowship in advanced higher education. The graduate school at Abertay University has been an extremely supporting and collaborative environment to undertake my PhD, and I thank the graduate school team for the training and support provided throughout my studies. The laboratory technical staff are an asset to Abertay university, and I thank this team for their input, guidance and expertise to make experiments happen.

I want to thank all the visiting research students and honours students I have met along the way. Many of you have provided fantastic assistance to large experiments and helping to enhance your training and experience in the Spiers research group has been a pleasure. I pay especial thanks to Emily Donaldson, our summer volunteer researcher. Your help and assistance during the summer months were immense. You have become a fantastic bench scientist and I hope you can continue your studies and follow a career in science. I thank our collaborators at the National Academy of Sciences, Ukraine. Elena and Olga our time spent together has been a highlight in my PhD, and my trip to Ukraine will be an experience I will never forget. I thank you both for your expertise and training, and your input in collaborative publications. I hope our paths cross in the future, and wish you both all the success in Kiev. I also thank Erasmus + for the funding to make this trip possible, and the Microbiology Society and the Society for Applied Microbiology for travel grants awarded to participate in conferences. To my fellow PhD students and ‘Book Club’ Thursdays - I wish you all well and success in finishing your degrees, and you have all brought laughter and support to this PhD journey. I especially thank fellow lab member Raji Bamanga for your collaboration within the laboratory, and for your support and training in the first few months of my PhD.

I wouldn’t have been able to consider perusing a career in science or undertaken a PhD without the support and guidance of my Parents, Janet and Neil. You have always put your children first and made me believe nothing is unachievable. I don’t

think myself and Shaun will every truly be able to repay you for both emotionally and financially supporting us through undergraduate and postgraduate degrees, allowing us to pursue our dream careers.

Finally, to my boyfriend Turtle. You have been there every step, for the wins and the failures, always with the right words or just a cold glass of wine.

Abstract

Static liquid microcosms have been used to study evolutionary and ecological dynamics of bacterial populations, where metabolic activity produces a heterogenous environment with a low-O_2 region in the liquid column, and a high-O_2 region directly below the air-liquid (A-L) interface. This system has been used to study adaptive radiation in *Pseudomonas fluorescens* SBW25, where non-biofilm forming wild-type populations diverge and biofilm-forming mutants known as Wrinkly Spreaders (WS) dominate the A-L interface where they are better able to exploit the ecological opportunity created by the high-O_2 region. Although this system is well understood, it is not clear why biofilm-formation is such a successful strategy for colonising the high-O_2 region. Similarly, this model system needs to be developed to reflect the complexity of microbial communities, where multiple species exist together and selective pressures may alter community composition, dynamics and emergent properties. Such better developed models can help understand changes in diverse natural occurring communities, enhancing our understanding of the progression of human infections, changes in ecologically important communities in agriculture, food production, and technology in applying microbial communities to mitigate the effects of anthropogenic pressures.

In this thesis I show that A-L interface biofilm-formation is the best strategy for colonising the high-O_2 region, where biofilms retain cells in position and overcome physical displacement including Brownian motion and bioconvection currents which otherwise would move aerotaxic wild-type cells away from the high-O_2 region. WS mutant cells also possess an additional mechanism to associate and penetrate the A-L interface, where the production of additional surface-active agents further lower surface tension allowing cells to break through the interface. Biofilm-formation can then initiate above the interface and explain the dry phenotype and strength of WS biofilms as the biofilm is situated at the air-side of the interface. These key adaptive changes of the WS mutant allows highly efficient cell localisation at the high-O_2 region to be achieved. However, biofilm strength is not directly related to fitness in static liquid microcosms. Other A-L interface biofilm-forming mutants within the SBW25 lineage can out-compete the WS mutant, producing weaker biofilms but maximising productivity by also significantly colonising the low-O_2 liquid column compared to the WS mutant, suggesting colonising both regions provides a fitness advantage in microcosms. Ecosystem engineering was further explored to fully capture the ecological dynamics of

diversifying SBW25 populations. Initial colonists not only generate O_2 gradients but they were found to further alter the chemical environment through the uptake of nutrients and production of secondary metabolites and toxic waste products, which effect the diversification, biofilm-characteristics and fitness of evolved WS mutants. This additional aspect to ecosystem engineering within the microcosms system was also reflected in community-level work.

I developed the microcosm model system for biofilm-forming communities using a soil-wash as the inoculum. The effects of heterogenous and O_2-limiting conditions on selection within bacterial communities were investigated and short-term serial-transfer experiments revealed changes in community productivity and biofilm characteristics. Productivity decreased in communities subject to longer incubation periods reflecting a tragedy of the commons with nutrient depletion and toxic waste-accumulation restricting growth. Final-transfer communities were stratified but retained phenotypic plasticity as isolates could form A-L interface biofilms as well as colonise the liquid column. Motility and cell localisation assays revealed isolates could migrate between both regions. This suggests a resource allocation trade-off between fast but competitive growth within the A-L interface biofilm and high-O_2 region and slower but less competitive growth in the low-O_2 liquid column, with community members maximising productivity by utilising the entire ecosystem.

My research contributes to the growing body of knowledge aiming to understand the evolutionary and ecological processes driving change in biofilm-forming populations and communities in static liquid microcosms. It shows the value of continuing to ask deeper questions surrounding biofilm-formation within model systems, and the importance of developing model systems to reflect the complexity of naturally occurring microbial communities. A-L interface biofilm-formation has been the main subject of interest within this research, however extending my focus to the liquid column has shown the importance of colonising the region below the biofilm to improve productivity, competitive fitness and community resilience. This suggests understanding the influence of non-biofilm space in biofilm-forming communities is important in understanding the complex dynamics and persistence of multi-species microbial communities.

List of Figures

List of Tables

Abbreviations

AM-WS: WA mutants evolved in aged media

CBA: Combined biofilm assay

CF: Cystic Fibrosis

CFU: Colony forming unit

CLSM: Confocal laser scanning microscopy

ds DNA: Double stranded Deoxyribonucleic Acid

eDNA: Extracellular Deoxyribonucleic Acid

EPS: Extracellular polymeric substance

FS mutant: *Pseudomonas fluorescens* SBW25 Fuzzy Spreader mutant

HCA: Hierarchical Cluster Analysis

KB-DA: Modified Kings B media double autoclaved

LPS: Lipopolysaccharides

OTU: Operational taxonomic unit

PCA: Principal Component Analysis

SEM: Scanning electron microscopy

ST: Surface Tension

Wild-type SBW25: *Pseudomonas fluorescens* SBW25

WS mutant: *Pseudomonas fluorescens* SBW25 Wrinkly Spreader mutant

Chapter 1

Introduction

Chapter 1. Introduction

Biofilms are a common mode of life for many microorganisms, a complex assemblage of cells and extracellular polymeric substances used to colonise biotic and abiotic surfaces. Biofilm research is dominated by molecular approaches, to understand biofilm-formation, structure and development. However, there has been a recent shift towards a more evolutionary and ecological perspective of biofilm research (Nadell, Xavier and Foster, 2009; Steenackers *et al*., 2016) to understand why biofilm colonisation is successful and found in a wide range on environments. Whilst eco-evolutionary biofilm studies are progressing, evolutionary and ecological content is still negatively correlated with molecular biology and medical content in reviews with a wider context of biofilm studies (i.e. the interests of biofilm researchers is still dominated by molecular biology and medical implications, and less on the broader eco-evolutionary interests, O. Moshynets and O. Lugin, Jerdan *et al*., 2020 In '*Bacterial Biofilms'*). Taking key evolutionary and ecological theories and concepts we can further our understanding of the establishment and progression of biofilm communities and determine the key eco-evolutionary dynamics that shape complex microbial assemblages. For this, single-species pure culture experiments must to be scaled up to reflect the environmental conditions and complexity of species diversity within biofilms in situ, consisting of multiple species competing and interacting. However, within biofilm research there are still fundamental questions surrounding why biofilm-formation is the dominate mode of bacteria life. Life within a biofilm presents clear advantages, including protections against physical disturbances, antimicrobial agents and predators (Flemming and Wingender, 2010). Biofilms forming at the air-liquid (A-L) interface provide cells with increased access to O_2, as demonstrated by diversifying populations of *Pseudomonas fluorescens* SBW25 (Travisano and Rainey, 1998; Koza *et al*., 2011).

Within this research I firstly aim to further understand the fundamental need for A-L interface biofilm-formation, exploring further ecological and evolutionary dynamics using a single-species microcosm system. While multi-species studies reflect the natural environment, they are complex, and single-species studies are still fundamental to understanding key aspects of biofilm-formation. I then aim to the develop this microcosm system to explore the impact of selection with O_2 limiting conditions on the productivity and biofilm-associated and community aggregates traits of a multi-species community. This will require knowledge of the appropriate methods for developing a multispecies biofilm system, key evolutionary and ecological processes underpinning selection and ecology and evolutionary dynamics shaping the success and fitness of individuals to succeed within a community.

Within this introduction I will explore the main evolutionary and ecological theory applied in microbial research and key eco-evolutionary dynamics that shape community emergent properties and the phenotypic and trait-based diversity in microbial communities. I will then explore biofilm-formation and development, with particular focus on A-L interface biofilms and the current knowledge obtained from model systems. This will also review methods used in biofilm research, and the current methods used to scale-up single-species studies to more complex multi-species studies, and the difficulties when doing so. Finally, I will analyse the current literature on the adaptive radiation of model bacterium *Pseudomonas fluorescens* SBW25 which results in a call of adaptive A-L interface biofilm-forming mutants known as Wrinkly spreaders. *P. fluorescens* SBW25 will be the model bacterium for this research, and by exploring gaps in our understanding within this system may provide further insight into the fundamental need for A-L interface biofilm-formation.

1.1 Microbial Ecology and Evolution

Microbes play a crucial role to life on earth, key to many ecological processes such biogeochemical cycling, and develop profound relationships with other living organisms. This includes the increasing rise of microbial infectious diseases in humans, which is estimated to surpass other killers such as cancer by 2050 due to the rise in antibiotic resistance (Zaman *et al*., 2017). Similarly, global warming and other anthropogenic pressures have a major impact on the biodiversity of microbial life, where loss in diversity has a serious impact on ecosystem function. Ever-changing environmental conditions subject microorganisms to continual selective pressures, requiring constant adaption (i.e., evolution). It is therefore important to understand the evolutionary and ecological processes that shape microbial communities. This will develop our understanding of the progression of human infections, changes in ecologically important communities, and application of microbial populations and communities in reversing the effects caused by human pollution.

1.1.1 Experimental evolution

1.1.1.1 Theory of natural selection

Charles Darwin’s theory of natural selection is and will continue to be one of the most profound areas of research leading to our understanding of the progression of life on earth. Darwin’s deductive and inductive approach led to the development of the theory of natural selection, following inspiration from Alexander von Humbolt to explore, collect, treasure and connect (Bowler, 1989). Darwin demonstrated natural selection through the diversification of

finch populations throughout the Galápagos Islands in South America. Populations became isolated on different Galápagos Islands, and Darwin reported differences in beak form and function (Darwin, 1859). Scottish geologist Charles Lyell also made crucial observations during this time, contributing toward the modern view of ecology. He questioned the old idea of a static, harmonious earth with the concept of “ecological niches” created through gradual changes in the environment. He concluded no organism could be ideally adapted to a single condition. Building upon this idea, Darwin hypothesised organisms constantly ‘struggle to exist’ (Bowler, 1989).

Natural selection theory was developed from three observable facts and two deductions (Huxley, 1942). All organisms have the ability to increase in a geometrical ration, but despite this, species populations remain relatively constant suggesting a competitive “struggle to exist”. Darwin observed vast variation between organisms, and between the Galápagos’ finches, and thus alluded to natural selection whereby only some variants are successful. Here, only offspring with characteristics suited to the current environment will survive. However, the original theory of natural selection does have weakness. Darwin only focussed on inheritable variation, thus his theory only included transmission of inheritable characteristics. He even stated in *Origin of Species* that non inheritable variation was irrelevant (Darwin, 1859). Darwin also believed natural selection was not observable in real-time. The long lifespan of organisms resulted in change occurring over hundreds of generations over hundreds of thousands of years, and so relied upon fossil records for any key proof to support his theory. The difficulty in providing clear ‘real-time’ experimental evidence lead Darwin to rely on artificial selection seen in the breeding of animals such as dogs and pigeons for evidence (Bucking *et al*. 2009). However, research which followed Darwin’s seminal work lead to the modern synthesis of evolution and creation of experimental evolution, where documented evidence of selection in real-time was first reported.

1.1.1.2 Modern synthesis and the rise of experimental evolution

After the publishing of *Origin of Species,* a unified body of work encompassing ecology, genetics, palaeontology, geography, embryology, systematics and comparative anatomy developed the modern synthesis of evolution (O’Malley, 2018). This developed the theory of evolution at the genetic and phenotype level, made possible with the advances in genetic technology. The modern synthesis identified four fundamental processes of evolution of both deterministic and non-adaptive forces (Svensson and Bergen, 2019). The first is the

deterministic process of selection, identified by Darwin in his theory of natural selection, where organisms best adapted to their environment survive and succeed, and as a result reproduce more (Darwin, 1859). The other three processes are non-adaptive, as they are not a function of fitness properties of an organisms, but influence selection. Recombination, resulting in the variation within and between chromosomes and mutations with DNA sequences is the ultimate cause in variation between individuals. This variation can be advantageous, and these individuals will be selected within the community, or can be deleterious and selected against (Figure 1.1.1). The fourth processes, genetic drift, is the random loss of genetic traits or an allele becoming widespread, independent of the survival or reproductive value of the trait, creating deviation between generations. These forces represent the fundamental forces of evolution (Lynch, 2007).

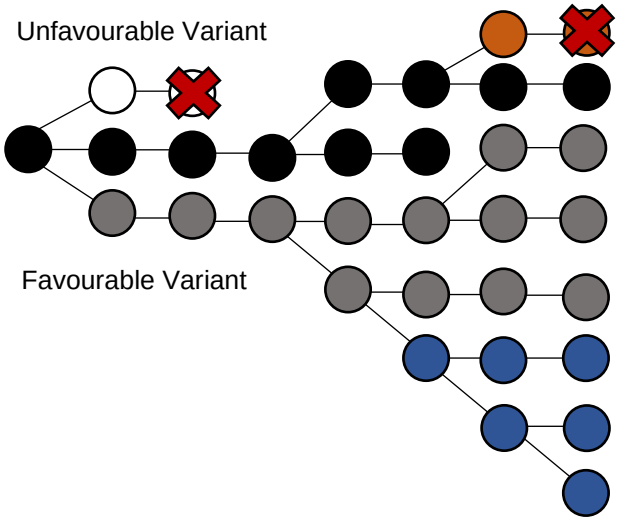


Figure 1.1.1 Deterministic and non-adaptive forces result in variation amongst individuals of a population. Mutations, recombination and genetic drift can introduce variation within a population, and these stochastic and non-adaptive processes influence the fitness or adaption of an individual's survival within the population. This variation can be favourable, and this variant is selected for and can reproduce. Other variants are unfavourable and are selected against.

The modern synthesis of evolutionary theory led the development of experimental systems to study evolutionary process within real-time. Many organisms have been used to study evolution, including fruit-flies and mice (Bell, 2007) but the most adept and documented are microorganisms, including bacteria, viruses, yeasts and protozoa. Microorganisms are easy to culture and can be cryogenically frozen allowing any given time-point of an experiment to be captured. A fast generation time and small size allows for 50 to 100 generations to be grown in just five days (Dykhuizen 1990), making for an ideal experimental organism. An

early concern of microbial use in experimental evolution was the lack of sexual reproduction. Microorganism are generally regarded as being asexual and reproduce through binary fission. The evolutionary theoretical framework was based on sexual reproduction, resulting in variation between offspring through reproductive recombination. However, the occurrence of mutations within populations of microorganisms provided a similar capacity for the introduction of variation as reproductive recombination through the high-frequency recombination between self-transmissible plasmids and host chromosome leading to portions of the genome are transferred between cells in a 'sexual' manner (O'Malley, 2018). William Dallinger led seminal work in experimental evolution and microbial evolution studies in 1874. Dallinger maintained flagellates for several hundred generations, while gradually increasing the temperature, resulting in populations adapting and thriving at temperatures far greater than those of the ancestor (Dallinger, 1874). Theodosius Dobzhansky further highlighted the value of microbial populations in experimental evolution due to short generation times and vast population sizes, and the rapid advances in genetics allowed mutations and selection to be easily studied (Dobzhansky*,* 1951*)*. Experiments showing the development of antibiotic resistant in populations of *Staphylococcus aureus* later highlighted the random occurrence of mutations and the ability for these mutations to be selected within the population (Stebbins and Ledyard, 1950). This work suggested that mutations and selection within microorganisms call lead to an evolutionary sequence, creating strains with new traits.

Experimental evolution is now defined as the study of evolutionary process, or element of, within a population in response to conditions imposed by the experiment (Buckling *et al*., 2009: Kawecki *et al.,* 2012; Steenackers *et al*., 2016). This allows new patterns in evolution and selection to be studied, or test existing theory. An appropriate system must be used allowing for defined parameters to be tested, genetic or phenotypic, to establish changes within the population or community. Many bacterial strains are now commonly use in evolutionary studies, in particular model strains of *Escherichia coli* and *Bacillus subtilis, Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Many of these strains are also prevalent in hospital infection or play key roles in ecological systems and plant growth promotion, so develop our understanding of the progression of acute infections and key ecological processes. The rise in experimental evolution transitioned evolutionary biology from a historic science to a dynamic science, in which evolutionary processes can be viewed in real-time (Buckling *et al*., 2009).

1.1.1.3 Key model bacteria in experimental evolution

Richard Lenski and colleagues were at the fore front of experimental evolution in the early 1990's, in their long-term *Escherichia coli* evolution experiments. Lenski explored the evolutionary dynamics of *Escherichia coli* B by serially transferring 12 replicate populations of the same ancestral strain every 24 hours. Initial reports displayed results over the first 2000 generations (Lenski *et al*. 1991), but this experiment continues today and has surpassed 65,000 generations (Lenski, 2017). This was the first study of its kind to show long-term evolutionary dynamics in real-time and over thousands of generations adaption and divergence has occurred within replicate populations. These changes are analysed by comparing cell morphology, mutations rates, genetics, competitive fitness and responses to environmental change (Lenski, 2017) between the ancestral strains and individuals from adaptive lineages across various time points.

The long-term *E. coli* evolution experiments follows a simple experimental design. Replicate populations of *E. coli* B were established from single haploid cells incubated in small flasks, with shaking incubation and medium containing glucose as a limiting resource. Every 24 hours 1% of the population is transferred to a fresh flask, and each day bacteria experience an initial lag phase followed by exponential growth through the uptake of limiting resources, and then enter the stationary phase. It is estimated that every 15 transfers sees 100 generations of *E. coli* B. Every 100 – 500 generations samples are cryogenically frozen to act as a fossil record and for direct comparisons with future generations (Lenski, 2017). Within the first 2000 generations the relative fitness of *E. coli* B increased approximately 40% (Lenski et al. 1991). After 10,000 generations cell size almost doubled, and this was consistent over all 12 populations (Lenski and Travisano, 1994; Lenski and Mongold 2000). At 30,000 generations metabolic diversity was observed in some replicate populations, utilising alternate carbon sources once glucose is consumed (Blount *et al.,* 2008).This experiment is a prime example of natural selection, however small differences in cell size or relative fitness between each of the 12 replicate populations indicate that chance and contingency also play a role in evolution (Buckling *et al*., 2009). This serial – transfer approach and comparison methods remain a key method for experimental evolutionary and ecological studies today (Figure 1.1.2).

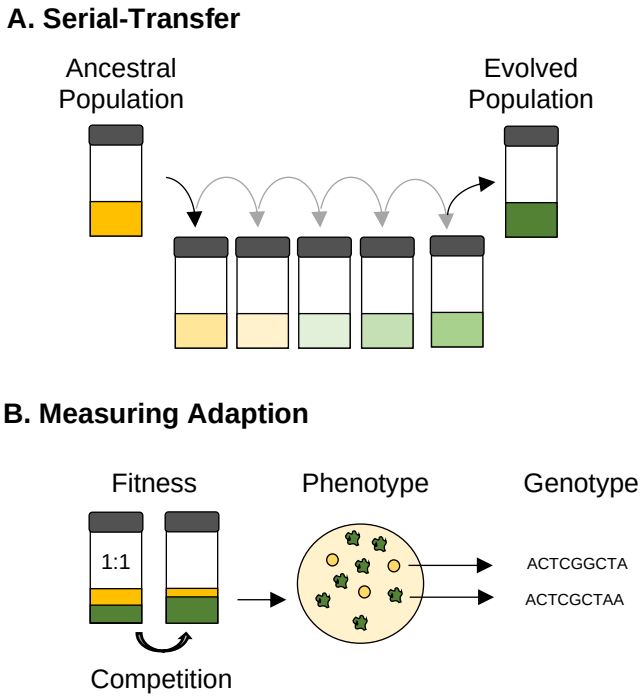


Figure 1.1.2. Serial-transfer approach of Richard Lenski's *E. coli* long-term evolution experiments. Long-term evolution experiments were started in the 90's by Richard Lenski and colleagues using a serial-transfer approach. Every day a subsample of each population is transferred to a fresh environment, representing a pulse disturbance or re-seeding event. To measure adaption and evolution samples from throughout the transfer experiments are saved and can be compared to one another and the original ancestral strain. This can include pair-wise competitive fitness comparison, changes in cell morphology and phenotype and genetic analysis to identify new mutations responsible for the changes observed. (Figure modified from Buckling *et al*., 2009).

Similar to the extensive research undertaken with population of *E. coli* B, the human pathogen *Pseudomonas aeruginosa* has also become a model organism in evolutionary studies. *P. aeruginosa* is of particular interest because of the involvement in chronic infections in cystic fibrosis (CF) patients, where thick mucoidal *P. aeruginosa* biofilms form in the lungs of CF patients. However, early research assumed cystic fibrosis *P. aeruginosa* strains were genetically similar, and so knowledge and diagnosis relied on a single isolate. Evolution studies and whole genome sequencing revealed the complex adaption and diversification of *P. aeruginosa* in cystic fibrosis infections (Winstanley, O'Brien and Brockhurst, 2016). Conditions within the CF lung are extreme, with many environmental stressors including osmotic stress (Brocker *et al*., 2012), high concentration of antibiotics through treatments attempts (Fodor *et al*., 2012) and oxidative and nitrosative stresses through host response (Hector, Griese and Hartl, 2014; Wood *et al*., 2009). In addition, a diverse range of microorganisms are competing within this environment, so *P. aeruginosa*

must adapt to survive. Studies following the evolutionary changes of *P. aeruginosa* through the progress of the chronic infections has revealed many evolutionary and adaptive changes, including the detection of hypermutators (Oliver *et al*., 2000). Mucoidal colonies have been observed through the increased production of alginate used for biofilm production, providing protection against antimicrobial agents and the host response (Bjarnsholt *et al.*, 2009), and individual cells show mutations that increase antibiotic resistance as a result of extensive antibiotic therapy (Breidenstein *et al.,* 2011). Other adaptions include the loss of motility for energy conservation, loss of virulence factors for progression to chronic infection, changes in metabolism and increased iron acquisition from haemoglobin, all resulting in *P. aeruginosa* becoming a sophisticated human pathogen (Winstanley, O'Brien and Brockhurst, 2016).

The evolutionary and genetic studies of *P. aeruginosa* not only progressed our understanding of how *P. aeruginosa* adapts and evolves through the progression of acute CF infections, but also provided key insight into the ecological and evolutionary dynamics of pathogenic microorganisms. Evidence of social traits including cheating and quorum sensing (Kummerli, 2015; Rumbaugh *et al*., 1999), and patterns in the evolutionary changes found in pathogen infections can be used as the basis for therapeutics for other infectious diseases and pathogens.

1.1.1.4 Microcosm system studies in experimental evolution

Microcosm model systems are commonly used in experimental evolution and have been historically used to study the ecological and evolutionary dynamics within microbial populations. As early as 1884, William Dallinger utilised microcosms to study temperature adaption of flagellates (Dallinger, 1874). Microcosm systems have been fundamental in developing our understanding of biofilm-formation (Winogradsky, 1887; Egunoy, 1895; Egunoy, 1926; Sorokina, 1938), and key evolutionary and ecological principals such as the competitive exclusion principle, stating no two species competing for the same niche can coexist (Gause, 1932). Microcosms have been criticised for their simplistic and highly artificial nature however, the intention of microcosms are not to mimic a specific environment, but rather capture the essence of an evolving system (Jessup *et al*., 2004; Buckling *et al*., 2009), and understand simple selection pressures e.g. O_2 availability.

Bacteria incubated within microcosm system begin to evolve and adapt to the environment in response to environmental stress and opportunity created within systems. Due to their small size and reproducibility, microcosms can be easily maintained over long durations of time

and manipulated to change environmental conditions such as temperature and nutrient availability. Multiple variables can be measured using an array of techniques differing in complexity (Altermatt *et al*., 2015). Analysis depends on the level of organisation and variables of interest, ranging from population studies of individual level behaviour or adaptive radiation, community ecology measuring species interactions, invasion or resilience and whole ecosystem productivity capturing nutrient and carbon cycling, energy fluxes or decomposition rates. Measurements take a simple approach such as optical density and colony counts to asses changes in growth rates, phenotypic and behavioural changes or interaction studies, to complex genomic sequencing and phylogenetic methods (methods extensively reviewed by Altermatt *et al.* 2015).

Microcosm studies are now common within microbial ecology and evolution, exploring many key dynamics and concepts including the relationship between diversity and productivity (Eisenhauer, Scheu and Josset, 2012; Brockhurst, Buckling and Gardner, 2007), the importance of negative and positive community interactions (Foster and Bell, 2012; Brockhurst *et al.,* 2010) and dynamics of microbial invasion (SheoPenj *et al.,* 2019). Microcosm are key model systems for the exploration of adaptive radiation and niche-construction theory (Rainey and Travisano, 1998; Brockhurst *et al.,* 2007) and exploring the underlying molecular biology and ecological dynamics of air-liquid interface biofilms (Spiers, 2014; Koza *et al.,* 2017). Despite the simplistic nature, microcosm systems are used to study systems of multiple species from natural microbial communities, studying important issues of global environmental change (Garnier *et al*., 2017).

1.1.2 Microbial Ecology

Microbial ecology is the study of microorganisms and their relationship with each other, the environment and their host. The ecology of microorganisms is a vital stream in microbiology, particularly within the initial identification of the role of microorganisms as infectious agents causing disease and role in processes such as fermentation. A period of pure culture followed, with the advances in molecular biology and genetic techniques microorganisms were studied in isolation in highly controlled laboratory settings. However, now many fields within microbiology continue to take a more ecological perspective to microbial research (Posser *et al*., 2007; O'Malley, 2014). As microorganisms are phylogenetically and physiological diverse, considerably more than macro organisms, the processes that underpin microbial interactions are much more complex (Posser *et al*., 2007). This is a challenging field of both contemporary ecology and microbiology, but the application of ecological theory

is invaluable to the understanding of the essential role microorganisms play in ecological processes, including biogeochemical cycling, and the progression of microbial infections.

1.1.2.1 Ecology

Ecology is the study of interactions among organisms and their biophysical environment. Interactions can be between organisms of the same species, between different species and between organisms and their environment. The main aim of ecology is to understand how interactions influence the density and diversity of organisms within an ecosystem, while studying how energy and matter flow through organisms and the environment (Stirling, 2002; Konopka, 2009).

There are four broad areas of research within ecology. Behavioural ecology concerns the behaviour or organisms which leads to survival, reproduction and population growth. Ecosystem ecology studies both the organisms and the environment, including interaction between the two. Population ecology focuses on population growth, where 'population' is defined as a group of individuals genetically or spatially distinct from others (Wells and Richmond, 1995). Finally, community ecology seeks to understand patterns in the diversity, abundance and composition of communities, and the process underlying these patterns (Vellend, 2010). This includes understanding the structure of communities, and how interactions between species influence community function and structure over space and time (Konopka, 2007). Community ecology also considers how abiotic factors influence species interactions or filters species which are present (Dunson and Travis, 1991).

Defining a 'community' has been a difficult debate within ecology, however, is generally accepted as an assemblage of more than one species, occupying the same geographical space at the same time (Konopka, 2007). Communities can be viewed as supra-organisms, where communities are considered as one functioning unit and interactions within determine community properties and function (Clements, 1916). However, others consider communities as co-occurring species which tolerate the same chemical and physical nature of the habitat, but do not necessarily interact (Gleason, 1926). Community ecology is a complex field, and the processes underlying community ecology dynamics are of specific interest to microbial community studies.

Key ecological patterns of species composition or diversity can be a result of numerous interacting processes and the uniqueness of each study system (Lawton, 1999). Similar to evolutionary theory, ecology is also governed by four main classes of ecological process (Vellend, 2010). The first is the deterministic process of selection. Fitness differences

amongst individuals determine succession of individuals within the community which includes response to the abiotic environment, type of interactions between species and functional traits of the individual. Within community ecology selection refers to fitness amongst species within a community, rather than individuals within a population, and the identity of an individual can be based on behavioural or functional traits rather than species identity (McGill *et al.,* 2006). Drift, the stochastic changes in species abundance, and speciation also change community structure dynamics by influencing species diversity and richness. Finally, dispersal influences species abundance and distribution, and is dependent on the size and composition of the community in which the disperser came from or is dispersing to (Holyoak, Leibold and Holt, 2005). Although over 120 different hypotheses have been identified to explain the maintenance of species diversity (Palmer, 1994), all mechanisms involve the four main ecological processes discussed.

The quantification of species diversity, richness and evenness is central to many ecological studies. Diversity refers to the number of different species within a community, and richness the number of individuals of a species. Species evenness measures how equal species abundance is within a community, where low evenness indicates dominant and rare species present within a community. The stability of ecological communities is influenced by all three factors, and variation in any can result in loss of biodiversity or can weaken community resistance or resilience (Ehrlich and Wilson, 1991). Calculating species diversity and richness is therefore important, where species indices are used. Many species indices exist for experimental quantification of diversity, but broadly fall under two categories; dominance indices and information-statistics indices (Stirling, 2002). Dominance indices are weighted towards species of higher abundance, including the Berger and Parker index (Berger and Parker, 1970) and Simpson index (Simpson, 1949). Most widely used is the Simpson index, which gives the probability that any two random individuals from an infinitely large community belong to the same species. Here, the Simpson index uses information from a broad array of species in the community so is more accurate than Berger and Parker index. However, dominance indices are heavily weighted towards the most abundant species. Information-statistic indices take into account rare species within the community and works on the principal that diversity can be measured similar to how information is contained in a code or message (Stirling, 2002). The outcome is therefore influenced by the number of species, and evenness. This includes the widely used Shannon index (sometimes referred to as the Shannon-Wiener index (Shannon, 1948; Spellerberg and Fedor, 2003). While other indices exist, the Shannon and Simpson index provide a general overview of species abundance and evenness within a community.

To quantify species diversity, a definition of what constitutes a species must be clear. The most common definition in ecology is that of Ernst Mayr, commonly known as the 'biological species definition' or 'biological species concept'. Mayr defines a species as 'A group of actually or potentially interbreeding natural populations, which are reproductively isolated from other groups' (Mayr, 1942). Although this definition has been extremely influential and is commonplace in many ecological textbooks, the definition does provide sources of disagreement. As a result, there are now many variations of the definition based on different biological properties including ecological (the occupation or distinct niche or adaptive zones), phylogenetic (concerning diagnostability or monophyly), evolutionary and biological (Queirox, 2005). Another issue in species quantification is individuals from the same species can exhibit different functional traits and behaviour which are of ecological importance. Recent advances in genomic identification methods suggest genetics and functional diversity are not necessarily mapped (Kraemer *et al*., 2010 and Vos and Vellicer, 2006). Therefore, recent research looks to quantify species diversity based on function rather than taxonomic diversity called functional, phenotypic or trait diversity (Fontana, Petchey and Pomati, 2016). This is receiving increased attention, and results suggest it is beneficial to relate ecological structure and changes within an ecosystem to function rather than taxonomic diversity (Tilman *et al*., 1997; Hillebrand and Matthiessen, 2009; Reiss *et al*., 2009).This also indicates the level of functional redundancy within the community. Functional redundancy or functional similarity is important in community resistance or resilience, where multiple members of the community are capable of carrying out a functional process, creating greater recoverability. High levels of functional redundancy allow communities to continue delivery of community function in response to environmental stressor or disturbance (Naeem S, Kawabata Z, Loreau M. 1998; Allison and Martiny, 2008).

1.1.2.2 Ecological theory in Microbiology

Microbial ecology combines the large and mostly macro organism focussed field of ecology and microbiology, through the introduction of ecological theory to microorganisms. As a result, areas of research within microbiology that were once dominated by molecular or genetic content are now taking a more ecological perspective, including the study and progression of microbial infectious (Magalhães *et al*., 2016), dental biofilm research (Marsh, 2018) and contamination of food processing facilities (Valderrama and Cutter, 2013). However, there are still inconsistencies of terminology and opposing views between community ecology and microbial ecology (Nemergut *et al*., 2013). The major challenge in the application of ecology in microbiology, is the asexual nature of most prokaryotes. However, ecological theory is developed from a macro organism perspective with

recombination through sexual reproduction and work on a large scale of space and time which does not translate to asexual single celled microorganisms. Theoretical concepts and framework in ecology is based on the introduction of variation within the population through genetic recombination during meosis, and the ecological species concept defines species as groups of interbreeding individuals (Posser *et al*., 2007). However, the high occurrence of mutations within microbial populations introduces variation.

Throughout the history of microbiology, laboratory techniques were based on pure culture research, with heavy focus on the medical and biotechnological applications. Very little research encompassed the ecology of microorganisms, and more importantly that microorganisms were social organisms living in multi-species communities. In the late 19th and early 20th century an increasing body of research argued that experiments should include more natural environmental conditions and sources of microbes from nature. This would generate new knowledge of bacterial physiology and interactions, and take microbial knowledge further than pure-culture studies (Stainer, 1951). Seminal work by Sergei Winogradsky earned his title as the founding father, and 'first' microbial ecologist (Stainer, 1951; Moshynets, Boretska and Spiers, 2013; Dowrking, 2017), with his use of elective cultures (now known as enrichment cultures) to study biological processes. Winogradsky emphasised that in order to study biological processes, communities of bacteria had to be studied rather than the single culture approach of his medical microbiology peers. Through his ecological approach, Winogradsky discovered nitrifying and sulphur oxidizing bacteria proposing bacterial chemolithotrophy and chemautotrophy, and he discovered the rich biodiversity of aquatic bacteria (Zavarzin, 2006). From Winogradsky's discoveries and ideas Winogradsky columns, a large microcosm system, were developed. Large glass columns contained pond water and soil to replicate a more natural environment, with the addition of carbon and sulfur source for nutrients. This provided a more 'in situ' system to study a large diversity of microorganisms. Within, numerous chemical and gas gradients establish, and microbial succession and community function can be observed and taught (Moshynets, Boretska and Spiers, 2013).

Within microbial ecology, social theory is extensively used to study interaction with microbial populations and communities (Shapiro and Dworkin, 1997; Shapiro, 1998). Bacteria are interactive organisms capable of collective activity and can exploit intercellular communication to facilitate their success and adaption. Investigating interactions between microorganisms is now commonplace in ecological and evolutionary studies, and provides insight to complex community dynamics of microbial communities (West *et al.,* 2006;

Stubbendieck, Vargas-Bautista and Straight, 2016). Defining interactions amongst microbes becomes challenging as the complexity of the community increases (Figure 1.1.3), however as microorganisms are easy to culture and manipulate communities can be simplified to determine the type of interaction between two or three members.

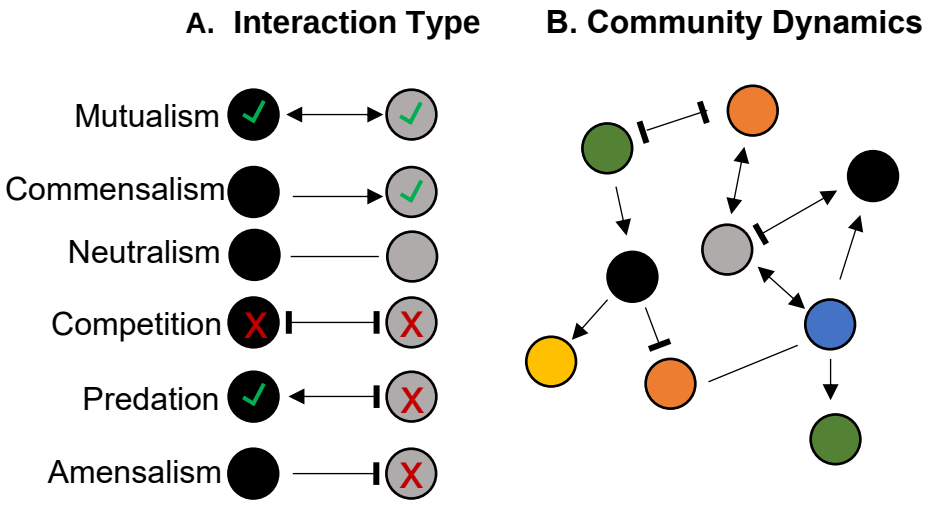


Figure 1.1.3. Identifying interactions between in microbial communities. Both negative, neutral and positive interactions can be identified between organisms (A). Green ticks represent individuals that benefit during the interaction and red cross represents individuals that are affected negatively. In pairwise comparison these interactions can be identified easily, however in complex communities identifying interactions is more difficult as many community members are interacting simultaneous (B).

Bacteria and other microorganisms live in communities, where a complex balance of positive and negative interactions occur. Bacteria can coexist without interacting, known as neutralisms. Here, species can be utilising different resources within the environment or occupy separate niche-space that contains the appropriate or optimal conditions. Microorganisms can interact symbiotically, where one or both species benefit from the interaction. In the case of mutualism, both interacting species benefit which can include cross-feeding between species where bacteria can exchange metabolites (D'Souza *et al*., 2018). This relationship can often become an obligatory relationship where one cannot exist without the other upon gene loss (Pande *et al.,* 2014). Commensalism is also common, where one species benefits from another. This includes the production of 'public goods', which are costly to produce but many members of the community or local population can benefit (Frank, 1998). Public goods can be products for nutrient acquisition including cell-cell signalling communication quorum sensing molecules (Parsek and Greenberg, 2005),

siderophores for iron chelating (Lee, van Baallen and Jasnen, 2016) and antibiotic or antibiotic degrading compounds (Riley and Wertz, 2002; Ciofu *et al*., 2000). Public goods can also provide sources for biofilm development and maintenance through the production of extracellular polysaccharides for the biofilm matrix such as alginate (Davies and Geesey, 1995) or adhesive polymers for attachment (Rainey and Rainey, 2003) However, commensalisms can open the community to exploitation. Here, cheaters take up shared resources, without giving anything to the community. This can result in instability or even collapse of the community. Common reported examples of cheating include the stealing of siderophore molecules or stealing of quorum sensing regulated processes in strains of *P. aeruginosa* (Popat *et al*., 2012; Griffin, West and Buckling, 2004)

Competition is prevalent in microbial communities and is often thought of as the dominating interaction amongst culturable microorganisms (Foster and Bell, 2012), and can be intra and inter specific. Competition effects both species involved, but in some cases known as predation or amensalism one species benefits from the interaction at the cost of another. Bacteria often compete for limiting resources such as nutrients and $O_{2,}$ and this can involve competition for niche space in which conditions are favourable. Recourse competition can be exploitation competition (Studdendiek, Vargas-Baustista and Straight, 2016) in which two species compete directly for a limiting resource trying to obtain more than the other species, or interference competition where one species will directly harm another (Stirling, 2002; Friedman and Gore, 2016). Here bacteria may release diffusible antagonists such as toxins or antibiotics to inhibit or kill a neighbouring species to defend territory or resources (Stubbendiek and Straight, 2016). Examples include oral bacterial communities where hydrogen peroxide is produced by *Streptococcus sanguinis* which inhibits growth of the competitor *Streptocccus mutans* in aerobic conditions (Kreth *et al*., 2008) or the production of the secondary metabolite pyocyanin by *P. aeruginosa* to kill neighbouring competitors increasing *P. aeruginosa* fitness in the CF lung (Lau *et al*., 2004). Bacteria can also adapt or evolve to exploit an environmental niche and better engage in exploitation competition. Mutations in *Pseudomonas fluorescens* SBW25 cells creates Wrinkly spreader mutants which can better exploit the high-O_2 rich layer in static liquid microcosms through air-liquid interface biofilm-formation (Rainey and Travisano, 1988).

Analysing interactions among species, and the functional pathways responsible, provide an ecological insight into the emergent properties of microbial communities. These characteristics and interactions can be captured by analysing component organisms in isolation as the network of interactions within larger communities can be extremely complex

to analyse and understand. Understanding the relationship and interactions between species provides the capability to predict system dynamics, and community response to environmental change (Konopka, 2009).

1.1.3 Eco-evolutionary Dynamics

Ecologists aim to understand how energy and matter flow through organisms and their environment and how the two interact, whilst evolutionary biologist are concerned with information on how organisms acquire or inherent the genetics that underpin their behaviour and adaption. Ecological interactions between organisms and the environment can influence the succession of species and dynamics within complex communities, while the acquisition of mutations changing individual species phenotype and fitness changing lineages over time is considered and evolutionary process. However, evolutionary and ecological changes are linked and can occur within the same space and timescale (Abrams, 2001; Pelletier, Garant and Hendry, 2009). Within bacterial populations eco-evolutionary dynamics are important in shaping the emergent properties within a community, and the phenotypic and genetic diversity within. Bacteria have short generation times and populations numbers are high, so selective pressures can result in extreme effects, and these effects can be seen over a short timescale.

1.1.3.1 Environmental modification

A major assumption in ecological or evolutionary biology is the abiota acts as a source of natural selection, however fails to address how organisms can modify the environment which in turn has ecological and evolutionary consequences (Konopka, 2009). Niche-construction, or niche-creation, and ecosystem engineering are central concepts identifying the effect of environmental modification, both on neighbouring individuals and to the modifying organism. This provides a link between evolution, ecology and ecosystems science (Mathews *et al.,* 2014). Environmental modification can be direct or indirect, have positive or negative effects, and can influence an individuals own evolution (Odling-smee *et al*., 2003, Laland, 2014). Although both niche-construction and ecosystem engineering have great similarities, niche-construction theory is considered to draw upon ecosystem engineering concepts (Odling-Smee *et al*., 2003) and both were developed independently with separate origins. Sometimes considered synonyms, ecosystem engineering used primarily in ecology, and niche-construction used in evolutionary biology (Boogert, Patterson, Laland. 2006). However, through extensive literature searching, differences do exist between the two definitions. I have set definitions based on the literature in Table 1.1.1.

Table 1.1.1 Definitions of environmental modification concepts based on the literature.

Concept	Definition	Sources
Niche-construction	A significant chemical and physical modification of the environment that influences the selection pressures of recipient organisms, which may be measurable in the form of an evolutionary response.	Laland, Balke and Feldam, 2016. Mathews *et al* 2014. Olding-smee *et al.,* 2003.
Ecosystem Engineering	The process in which organisms directly or indirectly alter the physical or chemical environment by modification, maintenance or creation of habitat. The changes in biotic and abiotic material affect the space in which other species live, and these effects can outlive the engineering organism.	Jones, Lawton and Shachak, 1997. Wright and Jones, 2006. Hastings *et al*., 2007

The definition of niche-construction remains broad, however is generally considered as the modification of the environment by organisms, which can alter the selection pressures within the environment (Laland, Blake and Feldman., 2016). Even minor and common processes can alter the environment or create environmental heterogeneity, creating a selection pressure (Laland, Matthews and Feldman., 2016). Organisms do not necessarily adapt to the environment, but rather modify environmental conditions that may influence its evolution trajectory (Lewton, 1983; Levins and lewontin 1985). The theory was postulated as an evolutionary process (Odling-Smee, 1988) and is now applied in evolutionary biology, however many studies focus on macro organism systems. Larger organisms demonstrate intentional niche-construction, through the creation of nests or burrows. Niche-construction can also be un-intentional through physical and chemicals alterations through metabolism or the creation of shade by plant leaves. The modified environment can then be experienced by descendants, known a ‘ecological inheritance’. A recent review states two main criteria for niche construction: organisms must significantly modify the environment, and these changes must then influence selection pressures on recipient organisms (Mathews *et al*., 2014). This is considered sufficient for the definition of niche-construction (Odling-smee, Erwin, Palkovacs., 2013) however some add there must be an evolutionary response in at least one recipient (Mathews *et al.,* 2014).

Microorganisms demonstrate several examples of niche-construction (McNally and Brown, 2015). The production and uptake of molecules and nutrients within the environment can have key effects of the fitness and composition of microbial communities or populations. The production of scavenging molecules to increase nutrient consumption, including proteases and siderophores, alter the chemical environment which may have diminishing effects on neighbouring species. However, detoxification and excretion of metabolic by-products can result in cross-feeding, where multiple species can reside together, and benefit from the niche-construction of neighbouring bacteria (Estrela, Trisos, Brown, 2012). The importance of metabolic interactions and the effect of niche-construction is even gaining attention within clinical microbiology studies (Estrela, Whiteley, Brown, 2014). With many variations of the definition of niche-constructions, the term is likely under-used in microbial studies, and the theory is limited in the teaching of ecology (Odling-smee *et al*., 2003, Laland, Boogert and Evans, 2014). However, further microbial experimental verification is leading to a prevalence of niche-construction within the fields of microbiology and microbial ecology.

Ecosystem engineering has been long recognised by ecologists, where simple processes such as energy and material uptake and the production of waste can impact the physical and chemical environment (Wright and Jones, 2006). The term 'ecosystem engineering' was created due to a lack of consideration of organisms modulating resources and habitat availability. This changes biotic and abiotic material, effecting the regulation of energy and mass flow, and trophic patterns (Jone, Lawton and Shachak, 1994). Ecosystem engineering can affect species distribution, abundance and ecosystem processes (Hastings *et al*., 2007) and is defined as alterations in the physical or chemical environment by modification, maintenance or creation of habitat (Jones, Lawton and Shachak., 1997).

Similar to niche-construction theory, most experimental evidence of ecosystem engineering is focused on macro ecosystems (Hastings *et al.,* 2007). Microorganisms demonstrate ecosystem engineering, and changes in resources required for microbial energy such as nutrient, carbon and electrons can be altered (Gutierrez and Jones, 2008). Microbes produce a range of molecules causing chemical changes, not limited to waste products and nutrient scavenging molecules (Firn and Jones, 2003). Microbes are extremely sensitive to the environment change and even small changes in temperature and pH as a result of ecosystem engineering can have a major effect on microbial processes (McClain *et al.,* 2003). However, the term 'ecosystem engineering' is limited with microbial literature, and is mainly used in soil, sediment and aquatic systems, including the microbial decomposition of phytoplankton which created O_2 dead zones in coastal waters (Rabalais and Nixon, 2002) or

heat transfer by microorganisms in soil leading to biogeochemical consequences (Gutierrez and Jones, 2006).

As metabolic processes in microbial populations and communities are inevitable, so too is environmental modification. I therefore suggest niche-construction and ecosystem engineering are important concepts to consider with studying microbial system, to give a more inclusive insight into factors contributing to eco-evolutionary dynamics and the emergent community composition and properties.

1.1.3.2 Adaptive radiation

Evolutionary processes are thought to occur over thousands of generations, which in larger organisms can take millions of years. A central evolutionary concept which encompasses ecology is adaptive radiation, the evolution of ecological diversity within rapidly multiplying lineages. Here, a single ancestor can differentiate into to multiple species which differ in traits capable of exploiting and occupying the new environmental habitats (Schutler, 2000). The success of new species, or adaptive mutants, is measured in fitness, comparing to the ancestor or other adaptive lineages. The theory of adaptive radiation was developed during the modern synthesis era lead by Simpson (1953), Lack (1974) and Dobzhansky (1951), and is extensively reviewed by Schutler (Schutler, 2000). Schutler states adaptive radiation is the ultimate result of divergent selection for specialisation to alternate resources. One of the most widely known examples of adaptive radiation is Darwin's Galápagos finches (Grant, 1986).

Adaptive radiation incorporates three main processes of ecological theory. First is the differentiation in phenotype as a direct result changes in habitat colonised or resources consumed. Divergence in phenotype can provide alternate fitness in the new environment. The second process is the result of phenotype divergence through resource competition. Competition is a driving force for members of a community or population to exploit new environments or resources. This is driven by ecological opportunity, where species can be in the right place at the right time or acquire new novel traits which can exploit a previously unoccupied niche or interact with the environment in a fundamentally different or unique way (Losos and Mahler, 2010; Yoder *et al.,* 2010). The final process of ecological speciation is the arise of new species in the population through divergent natural selection from the environment and competition for resources (Schutler, 2000).

Many examples of adaptive radiation are found in microbial populations incubated in static liquid microcosms. Static liquid microcosms allow for resource heterogeneity to develop through O_2 or nutrient consumption (Maclean, 2005). Theory suggests that shared resources in monomorphic populations generate adaption for alternate resources, and metabolic adaptive diversification has been found in populations of *E. coli* B (Friesen *et al*., 2004) and *Pseudomonas fluorescens* SBW25 (MacLean, Dickson and Bell, 2005) for different carbohydrate and carbon substrates. Adaptive radiation by niche-construction is also found in *P. fluorescens* SBW25, where niche-construction through the depletion of O_2 in the lower liquid column creates ecological opportunity at the A-L interface region where O_2 remains high (Koza *et al*., 2017). An evolutionary response results in Wrinkly Spreader mutants arising and dominating the population through exploitation of the high-O_2 conditions directly below the A-L interface through biofilm-formation. This results in a competitive fitness within the population.

1.1.3.3 Fitness

Fitness (often denoted as W) is a quantitative assessment of selection and success of a genotype or phenotype in a given environment. Fitness is influenced by both ecological and evolutionary dynamics, including changes in genotype through the acquisition of beneficial or deleterious mutations, changes in the environmental conditions, and the genotypes of neighbouring strains within the community. Fitness can be calculated as the absolute fitness, defined as the proportional change in abundance of a genotype over one generation, or relative fitness, the change in genotype frequency. Commonly, in laboratory experiments of bacteria relative fitness is estimated as the change in frequency of strains grown together, often referred to as ‘pairwise’ competition assays (Dean, Dykhuzien and Hart, 1986; Lenski and Travisano, 1994). This is determined as the ratio of Malthusian parameters (mA/mB), where m = *In* [final numbers/ initial numbers] of population A and B, and m is scaled for generation time using *In* as a correcting factor (Lenksi *et al*., 1991; Chevin, 2011) and is often approximated as a ratio of the number of doublings of each strain or the relative growth speed (Wiser, Ribeck and Lenski, 2013). A competitive fitness (W) greater than one suggests that strain has a competitive advantage over the other, termed an adaptive or beneficial genotype (Van den Bergh *et al.,* 2018). This can be used to determine the competitive fitness between two different strains or species, or individuals of the same lineage by using the ancestral and evolved population, to determine changes in fitness as a result of adaption.

Increasing fitness is not always a beneficial strategy to future generations of the same species. Selfishness in an individual's own and immediate fitness can affect future longer-term success of that lineage, which is an example of 'The tragedy of the commons' (this term can also often be used to discuss with impact of cheaters in a population, Hardin, 1968). This is often found in microbial populations where initial colonists quickly deplete the environment through nutrient and O_2 consumption increasing fitness, at the cost of future generations left to survive in a degraded environment ultimately resulting in inhibition of growth (Maclean, 2008; Koza *et al*., 2017). Constant adaption even within a population is needed to remain fit, known as the red queen hypothesis (Liow, Van Valen and Stenseth, 2011). However, the fittest individuals do not always prevail in microbial communities, even when the selective pressure is strong. Second-order selection states that an unfit individual could outperform the strongest individual of the community, if it can acquire a beneficial mutation, or if environment conditions were to change, increasing its evolutionary potential (Van den Bergh *et al*., 2018).

Environmental conditions influence selection of phenotypes during evolution and ecological experiments. Under conditions such as chemostats, where conditions are maintained constant overtime, specialists are believed to be selected. Specialists have high fitness within the current environment but demonstrate trade-offs in others. Trade-offs can result from neutral mutations that are selective in one environment, but become deleterious in another, or where adaption to one condition is maladaptive in another (Van den Bergh *et al*., 2018). This was shown in the *Escherichia coli* B long-term evolution experiments where optimal growth of adaptive mutants grown on glucose became dependent on the presence of citrate for iron-chelating and on the temperature used in the experiment (Leiby, Harcombe and Marx, 2012, Cooper, Bennet and Lenski, 2000).This is also well known in viruses, with regards to host specificity (Elena, 2002). Trade-offs are also found in the form of resource allocation trade-offs where cellular resources are allocated to one characteristic which can lead to reduced fitness in another (Ferenci, 2009), often as a consequence of environmental constraints (Tilman, 2000). This includes trade-offs between nutrients and energy needed for attachment and biofilm development and energy that could be utilised for cell replication and motility (Bachman *et al*., 2017; Arcerenza, 2016). This may result in lower fitness or growth rates initially, but biofilm development increases fitness over a longer time period. Similarly, tolerance to environmental stressors such as heavy metals or pH (Porter and Rice, 2013) and changes in nutrient utilisation or metabolism (Fercini, 1996; Eames and Kortemme, 2012) can all effect growth rate and fitness.

Within changing or fluctuating environments, such as a serial-transfer approach, selection for generalists is predicted (Van den Bergh *et al*., 2018). This comes at a cost of lower fitness than specialists in separate conditions, however overall fluctuation in fitness is minimalised and individuals are more adapted to changes in the environment or unexpected stresses (Karve *et al.,* 2015). This is similar to phenotypic plasticity, the ability of taxon to develop and express different phenotypes in response to distinct environmental conditions (Pigliucci, 2001), and is a mechanism to escape extinction through changes in local conditions (Chevin *et al*., 2010). This has been demonstrated in marine microbial communities using continuous cultures, where phenotypic plasticity stabilised community functional response to environmental changes (Beier *et al*., 2015). A similar response to fluctuating environments and ensuring long-term success is bet-hedging. Bet-hedging is a risk-spreading strategy evolved in unpredictably changing environments (Jong *et al.,* 2011), where isogenic population maximise mean fitness across various environments by minimising the temporal variance of surviving offspring (Grimbergen *et al*., 2015). This multi-stable response is a stochastic switching between phenotypic traits and is thought to be among the earliest evolutionary solutions to life in fluctuating environments (Beaumont *et al.,* 2009). Bed-hedging was developed in 1970's as part of evolutionary theory (Slatkin, 1974; Gillespie, 1974) however like many evolutionary concepts it was created with multi-cellular sexual reproductive organisms in mind so challenges exist in identifying bet-hedging in microbial populations (Jong *et al*., 2011). Many microbial examples have been identified including the stochastic sporulation of strains of *Bacillus subtilis.* Strains begin sporulation without initiation from the environmental cue of depleting conditions, which ensures survival if environmental conditions suddenly change and is too late for sporulation to take effect (Veening *et al*., 2008; Siebring *et al.,* 2014). All strategies are a response of survival, where bacteria seek to increase overall fitness within the community and succeed.

1.1.4 Concluding Remarks

A central concept in microbial studies is to study the adaption and survival of microorganisms, and the underlying processes that shape complex communities. A number of evolutionary and ecological processes, both deterministic and stochastic, can be responsible for the success, survival and adaption of microbes in an everchanging environment. Both from evolutionary and ecological perspective these processes determine the diversity and traits of bacteria present in microbial communities, and the variation found between individuals of the same species. Microbes are social entities which interact in both competitive, neutral and positive ways, and these interactions also effect the emergent community properties and survival of species within. By understand the eco-evolutionary

processes and dynamics occurring within microbial populations and communities we can develop our understanding of how to tackle the rise in infectious disease and restore ecologically important microbial communities within the environment.

1.2 Biofilms

Many microorganisms, including bacteria, form and reside within biofilms in order to colonise and inhabit both biotic and abiotic surfaces (O'Toole, Kaplan and Kotler, 2000; Hall-Stoodley and Stoodley, 2002). Biofilms consist of densely packed cells surrounded by a self-produced matrix of excreted biopolymers, polysaccharides, proteins, nucleic acids and lipids (Nadell, Xavier and Foster, 2009). The extra-cellular polysaccharide matrix provides cells with protection against physical disturbances, antimicrobial agents and predators (Flemming and Wingender, 2010), suggesting life within a biofilm is superior to planktonic (free-swimming cells) state. Biofilms can consist of populations derived from a single species to complex multi-species communities, where synergetic and competitive interactions shape the composition and emergent biofilm properties. Although difficult to quantify, it was recently estimated that between 40-80% of bacteria and archaea live within biofilms (Flemming and Weurtx, 2019). Bacterial biofilms play a key role in many ecological processes such as biomass conversion, however, have a fatal role in hospital infections, emphasising the need for further fundamental biofilm research. It is widely recognised that biofilm-formation is the main and natural mode of life for most microorganisms (Parsek and Singh, 2003; Hall-Stoodley, Costerton and Stoodley, 2004), therefore fundamental research in biofilm-formation and development is needed to understand the key role microorganisms play in ecological processes and hospital-inquired infections.

Seminal work described biofilm samples from the human mouth as ‘aggregates of microbes’ by Antony van Leeuwenhoek in 1684 (Dobel, 1960). Later in 1922 Louis Pasteur observed similar aggregates in acetic wine where he described a slimy material throughout multiplying clumps of cells, termed ‘Mother of Vinegar’ (Pasteur, 1922), now known as the extracellular polysaccharide substances (EPS) which make up the biofilm matrix. Many studies utilising microcosm systems followed, with descriptions of gel-like material encasing bacterial cells termed ‘bacterial plates’ or ‘bacterial films’ forming on submerged surfaces and at the interface between liquid and air (Winogradsky, 1887; Egunoy, 1895; Egunoy, 1926; Sorokina, 1938). Many described bacterial cells within aggregates as sessile and not ‘free-floating’ like planktonic bacteria cells (Egunoy, 1926; Henrici, 1933). The term ‘biofilm’ was likely first used in the studies of ZoBell and Allen in 1935, where glass slides submerged in seawater resulted in bacteria cells adhering and subsequently biofilm developed (ZoBell and Allen, 1935). Later in 1977 the link between chronic infections and aggregates of bacteria was made, and images of biofilms of *Pseudomonas aeruginosa* strains were pictured on the lungs of a deceased cystic fibrosis (CF) patient. The images showed aggregated bacteria cells covered in thick slime in the sputum of CF patients (Høiby, 1977). Soon after, the first

use of the word ‘biofilm’ was published in medical reports by dentists (Jenderesen and Glantz, 1981 a). Biofilms are now considered ubiquitous in nature and infections, and are often described as ‘City of Microbes’ (Watnik and Kolter, 2000).

Pathogenic bacterial biofilms pose a huge threat in hospital acquired infections, with biofilm colonisation and development prevalent in immunocompromised patients, and hospital and surgical equipment. Approximately 80% of the infectious and persistent bacteria in hospital infections are biofilm-formers (National Institute of Health, 2002). Surgical equipment, catheters and implantable medical devices are regularly contaminated causing human infection when used, costing the health-care system thousands of pounds each year (Chen, Yu and Sun, 2013). Biofilms form on human teeth, referred to as plaque, and are the leading cause of most dental issues including cavities, gingivitis and periodontitis (Takenaka, Ohsumi and Noiri, 2019). Biofilms also cause serious problems in the food processing industry, resulting in food spoilage and economic loss for producers, and further strain on the healthcare system through consumption of food-borne pathogens (Coughlan *et al*., 2016). Pathogenic bacteria such as strains of *Escherichia coli*, *Listeria monosytogenes* and *Salmonella* spp. attach and form biofilms on food surfaces and on equipment such as vats and tanks in the food processing environment or production line, resulting in the contamination of consumables (Shi and Zhu, 2009). It has also been found that some pathogens, in particular *E. coli* O157:H7 and many *Salmonella* spp. biofilms can survive the disinfection treatment of plant-based food (Takeuchi *et al*., 2000). The economic loss through biofilm contamination requires further knowledge of preventative approaches to limit biofilm colonisation.

However, biofilms also have great biotechnological applications where the enhancement of biofilm growth and activity is advantageous. The diverse range of metabolic activity in bacterial species results in the degradation of organic matter, environmental pollutants such as heavy metals and petroleum, and the cycling of nitrogen and sulfur. As a result, bacterial biofilms have a wide range of applications in the industrial, environmental and agricultural industry (Davey and O’Toole, 2000). Biofilms play key roles in the processing of sewage and waste-water treatment, and the cleaning of contaminated ground water or land known as bioremediation (Boer *et al*., 1991; Massol-Deya *et al*., 1995). Microbial biofilms can also restore conventional agricultural soils through nitrogen fixation. The over-use of agrochemicals and fertilisers has had a detrimental effect on soil microbial communities resulting in a negative effect on soil health, fertility and therefore crop productivity (Senevirante *et al*., 2011). Research aims to restore the microbial health of soils, where the

introduction of synergistic biofilm-forming species would be beneficial. Similarly, the robust and diverse nature of microorganisms allows biofilms to form in extreme and niche environments, such as ice-covered lakes in which they play a vital role in biological processes such as photosynthesis, nitrogen fixation and fermentation (Paerl and Priscu, 1998). Although bacterial infections of plants can have a negative effect on plant health, biofilm-formation in the phyllosphere, rhizosphere and within plant tissue can significantly improve plant health (Morris and Monier, 2003). Growth promoting bacterial biofilms can simulate root growth, are involved in plant stress control and act as biofertilisation and rhizoremediation (Lugtenberg and Kamilova, 2009). Many fluorescent pseudomonads have applications in the promotion of plant growth (Wiehe *et al*., 1996; Fukui *et al*., 1994; Preston, Bertrand and Rainey, 2008).

With the degradation of our environment through anthropogenic pressures biofilm research is vital to understand mechanisms and methods of reinforcing biofilm productivity to enhance application in many agricultural and environmental settings. Anti-biofilm therapeutics also continues to seek methods of disrupting and preventing biofilm-formation in medical and industrial settings (Verderosa, Totsika and Fairfull-Smith, 2019), made even more challenging by the rise in bacterial antibiotic resistance. Research therefore continues to understand the fundamental aspects of biofilm-formation and development, and further investigate the ecological and evolutionary dynamics which can enhance our understanding of the progress of acute infections and the role biofilms play in key ecological processes.

1.2.1 Biofilm-formation and development

Biofilms can form at air-liquid (A-L), solid liquid (S-L) and air-solid-liquid (A-S-L) interfaces. Although these interfaces differ, the initiation of biofilm-formation and many other biofilm characteristics are very similar. Seminal reviews of biofilm development describe biofilm-formation and development as a series of linked events, producing a conceptual model (Costerton *et al*, 1994; Costerton *et al*., 1995). The concept of biofilm-formation was influenced by the developmental process of *Myxococcus xanthus* fruiting-body formation (O'Toole *et al*., 2000). Research which followed supported the overview that significant changes in the spatiotemporal organisation of individual cells results in the transition of free-swimming cells into a surface-associated microbial biofilm (Monds and O'Toole, 2009). The model of biofilm-formation demonstrates the distinct transitional phases throughout the biofilm developmental life cycle (Figure 1.2.1). This was developed from S-L interface biofilms of gram-negative proteobacteria. Critics highlight the lack of experimental evidence to support the stages of the developmental process (Monds and O'Toole, 2009) and the

perceived relevance to all types of bacterial aggregations (Moshynets and Spiers, 2016), however this model continues to be used throughout biofilm research.

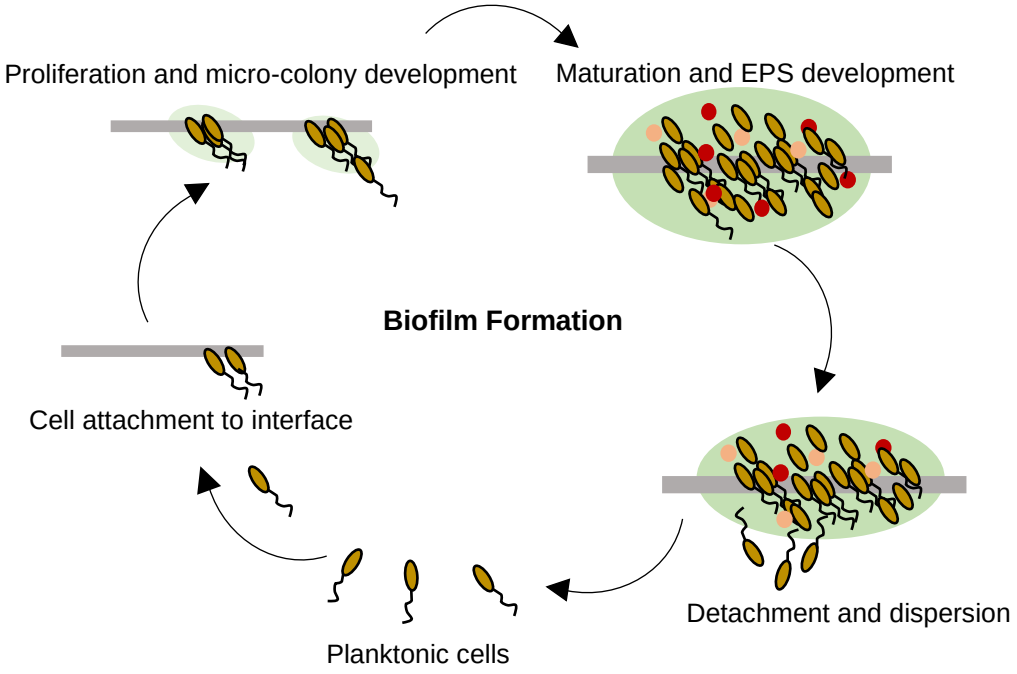


Figure 1.2.1. Initial conceptual model of the lifecycle of biofilm-formation. From initial reviews of microbial biofilm research (Costerton *et al*., 1995; O'Toole, Kaplan and Kolter, 2000) variations of this schematic is commonly used throughout biofilm research. The schematic demonstrates the lifecycle of biofilm-formation as a series of distinct transitions initiated by a response to environmental stimuli. Planktonic cells attach to an interface which is followed by micro-colony development. Cells then transition to life within a biofilm which initiates the production of extracellular polymeric substances which contributes to biofilm structure. This lifecycle ends within the detachment and dispersion of cells which transition back to planktonic free-swimming cells.

Biofilm-formation is initiated by the attachment of free-floating planktonic cells to a surface in response to changes in the environment or stimuli such as pH, O_2, osmolarity, iron etc. (O'Toole, Kaplan and Kotler, 2000). Once attached, biofilm maturation is commonly a linear process, particularly in *pseudomonas* spp. Free-swimming cells attach and continue to replicate, and through signalling molecules such as quorum sensing biofilm development begins (Hall-Stoodley and Stoodley, 2002). After biofilm maturation cells eventual disperse completing the developmental lifecycle, or the biofilm collapses. Biofilm-formation can also occur with the binary joining of micro-colonies, where daughter cells join up from multiple adjacent attachment points (Stoodley, Hall-Stoodley and Lappin-Scott, 2000; Hall-Stoodley

and Stoodley, 2002). During biofilm maturation cells within adapt to life within the biofilm by the secretion of extracellular polymers contributing to an extracellular matrix. Biofilm-formation can vary in timescale, depending on both biotic and abiotic factors. Biofilms can range from a structurally complex, thick and well attached biofilm, to very thin simplistic films consisting of very few cells and matrix components. Many genetic pathways have been found for biofilm development, and some are more common than others suggesting some pathways are not unique to species or environment (Moshynets and Spiers, 2016). A common pathway is the signalling network involving the secondary messenger cyclic-di-GMP. Cyclic-di-GMP is one of the most widespread messengers regulating biofilm-formation in bacteria, responsible for the transition between planktonic and biofilm life, and thought to be responsible for the progression of infections from acute to chronic (Römling, Galperin and Gomelsky, 2013).

The biofilm matrix of extracellular polymeric substances (EPS) is often referred to as the "dark matter of biofilms" (Whitchurch *et al*., 2002) due to the large range of biopolymers, complexity in analysis and high variability of EPS make-up between species. The EPS can account for up to 90% of the dry mass of a biofilm (Flemming, Neu and Woznaik, 2007) and can also consist of extracellular DNA which can have an important role in initial biofilm establishment (Nivens *et al*., 2001; Whichurch *et al*., 2002). The type of biopolymers produced is dependent both on the species present and the environmental conditions. Cells within the biofilm matrix are in close proximity leading to complex interactions from cell to cell signalling and retention of enzymes and DNA (Flemming, Neu and Woznaik, 2007). This provides opportunity for horizontal gene transfer, which can help increase antibiotic resistance of bacterial cells. The EPS itself provides cells with protection against antimicrobial agents and other biotic and abiotic disturbances including heavy metals, UV radiation, desiccation and predation (Elarsi and Miller, 1999; Almås, Mulder and Bakken, 2005; Martz *et al*., 2005; Burmølle *et al*., 2006; Change *et al*., 2007). This suggests cell survival is increased within a biofilm, compared to planktonic state, making the dark-matter of biofilms a key target for anti-biofilm therapeutics.

1.2.3 Air-Liquid Interface Biofilms

Liquid-solid biofilm model systems are a main research focus for their role in human infections. However, air-liquid interface biofilms also occur in a wide range of environments across many species. Although bacterial aggregations are now commonly termed 'biofilms', vast terminology is used in various research fields to describe biofilms forming at the A-L interface. 'Pellicle' is commonly used, particularly in the study of *Bacillus* spp. and

Shewanella oneidensis A-L interface biofilms (Kovács and Dragoš, 2019; Laing *et al*., 2010). Within applied and environmental microbiology 'microbial mats' is more common (Moshynets and Spiers, 2016). 'Rafts' or 'floating biofilms' (Armitano, Méjean and Jourlin-castelli, 2014) describes A-L interface biofilms not attached to any solid surface (Armitano, Méjean and Jourlin-castelli, 2014; Moshynets and Spiers, 2016). However, all are used to describe bacterial aggregations forming at the interface between air and liquid.

A-L interface biofilms can be found in sea or fresh water across a range of conditions (Wotton And Preston, 2005) and the ocean-air interface is often thought of as one giant gelatinous biofilm (Kjelleberg, 1985). There are many applications for A-L interface biofilms, in particular for the bioremediation and treatment of waste-water and contaminated waters (Ramana, Tomar and Singh, 2000). A-L interface biofilms are common amongst many species of bacteria, including strains of *Bacillus cereus and Bacillus subtilis* (Wiijman *et al*., 2006; Branda *et al*., 2001), *Salmonella entertidis* (Solano *et al*., 2002), *Escherichia coli* UPEC and O5:H7 (Weiss-Muszkat *et al*., 2010; Lim *et al*., 2012) and many species with the *Acetobacter* and *Vibrio* genera (Moonmangmee *et al*., 2002a; Deeraksa *et al*., 2005; Fong and Yildiz, 2007; Visick *et al*., 2013). One of the most common genus found to form A-L interface biofilms are pseudomonads. *P. fluorescens* SBW25 (Rainey and Travisano, 1998), *P. putida* KT2240 (Bridier, Piard and Bouchez, 2019) and *P. aeruginosa* PA01 and PA14 (Boles, Thoendel and Singh, 2004; Flynn *et al*., 2016) can form A-L interface biofilms, and surveys of plant, phytopathogenic, soil and river, and psychritophic spoilt meat-associated pseudomonads demonstrates A-L interface biofilm formation is very common amongst this genus (Ude *et al*., 2006; Robertson *et al*., 2013).

Formation of A-L interface biofilms is beneficial for aerobic bacteria as it allows access to high-O_2 conditions. A-L interfaces have opposing gradients, with downward diffusion of O_2 and upward diffusion of nutrients (Armitano, Méjean and Jourlin-castelli, 2014; Moshynets and Spiers, 2016). There are two possible hypotheses for the initial development of A-L interface biofilm development in laboratory conditions (Figure 1.2.2). The first predicts initial attachment to the wall of the vial or container, which leads to cells spreading across the surface of the interface. The second suggests cells aggregate randomly along the interface, which spread and join. The second is likely for 'floating' A-L interface biofilms, but either could depend on the conditions and species or strain (Armitano, Méjean and Jourlin-castelli, 2014).

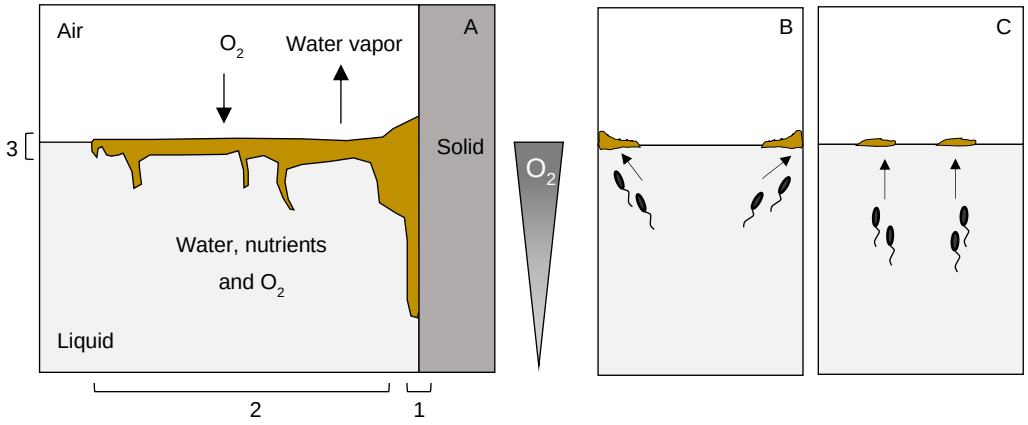


Figure 1.2.2. Conditions and development of air-liquid interface biofilms. A-L interface biofilms form at the interface between air and liquid (A, 3), and are often attached to a solid surface in microcosms vials (A, 1). At the interface there is opposing O_2 and nutrients gradients, with the downward diffusion of O_2 and upward diffusion of nutrients. Initial attachment occurs either at the vial wall (B), or randomly along the interface (C), after which biofilm development results in cells spreading across the surface of the interface. The biofilm matrix and cell interactions hold cells in place (A, 2). Figure modified from Armitano, Méjean and Jourlin-Castelli, 2014 and Moshynets and Spiers, 2016.

Cells require access to the A-L interface for biofilm-formation to occur. Under static conditions gravity and Brownian motion force cells down and away from the interface. This require cells to possess an additional mechanism to allow colonisation of the A-L interface. An important trait is swimming motility, with species shown to utilise aerotaxis to position cells at the A-L interface (Hölscher *et al*., 2015), and the flagella has also been evidenced to act as an anchor for initial attachment (Guttenplan and Kearns, 2013). Other traits include buoyancy, though the capture of CO_2 bubbles in *Gluconacetobacter* spp. (Zara *et al*., 2005). Once positioned at the interface, cells must possess traits to interact with the interface in order to attach, adhere or penetrate (Preston and Roger, 2005). This can include adaption in the form of fibrillar structures, or organic molecules and proteins which can lower the interfacial tension of the interface or promote hydrophobicity (Kjelleberg, 1985). Cells at the A-L interface then switch from motile planktonic state, to sessile EPS producing cells (Kobayashi, 2007). A wide variety of EPS can be found in A-L interface biofilms, which differ between environmental conditions and species. Common EPS components of A-L interface biofilms include cellulose, curli, lipopolysaccharides and eDNA (Armtianto, Méjean and Jourlin-Castelli, 2014).

Experimental evolution and ecological studies utilising wild-type non-biofilm forming model bacterium in static liquid microcosms have found populations rapidly diversify in response to

an O_2 gradient, resulting in adaptive mutants capable of A-L interface biofilm-formation arsing within the population. These model bacteria include strains of *B. subtilis, P. aeruginosa* and *P. fluorescens,* and the resulting adaptive mutants produce A-L interface biofilms in static liquid and produce wrinkled colonies on agar plates (Boles *et al*. 2004; Flynn *et al*. 2016; Koza *et al*. 2017; Kovács & Dragoš, 2019). Access to the A-L interface through biofilm-formation provides increased O_2 access, and within static liquid microcosms provides a fitness advantage over non-biofilm ancestors by blocking competitors and preventing further O_2 from diffusing into the liquid column, increasing the O_2 gradient throughout the liquid column (Loudon *et al*., 2016; Koza *et al*., 2011). A-L interface biofilm-forming mutants are considered adaptive and beneficial phenotypes in static liquid microcosms.

Initially, A-L interface biofilms were thought of as a specialist and rare form of biofilm, however growing research suggests many species have the genetic capacity to form A-L interface biofilms and is becoming an increasingly important research model. The A-L interface biofilm model system does have limitations, as often A-L interface biofilm-formation is occurring in response to specific conditions of the laboratory set-up (Kovács and Dragoš, 2019) and it still remains unclear where some A-L interface biofilm occur in nature. However, A-L interface biofilm-formation provides a system for experimental evolution and microbial ecology studies to study microbial interactions and fitness within a spatially structured environment (Koza *et al*., 2017; Kovács and Dragoš, 2019).

1.2.3 Multi-Species Community Biofilms

The axenic study of single-species are fundamental to the development and understanding of microbiology, and most of our knowledge of biofilms have come from single-species experimental models (Lenski, 2017). However, these studies fail to address the more realistic picture of microorganisms living within complex communities. Within nature, biofilms are complex assemblages of multi-species microorganisms, and life within a biofilm often results in cells from multiple species living within close proximity (Hall-Stoodley *et al*., 2004; Elias and Banin, 2012). This provides ample opportunity for intra and interspecies interactions, and these interactions can shape the emerging functions and properties of the biofilm (Friedman and Gore, 2016). Single-species studies need to be scaled up to reflect community level complexity (Lenski, 2017). Community-based biofilms (multispecies/ poly-species) are often more resistant to antimicrobial agents and invasion compared to single-species biofilms (Burmølle *et al*., 2006), and can even increase virulence in infections (Lopes *et al*., 2013; Pastar *et al*., 2013). The study of multi-species biofilms therefore provides a

more realistic scenario of the formation and progression of biofilm infections. Community biofilms also have increased application in biotechnology and industry, as more complex communities show increased organic compound degradation (Yoshida *et al*., 2009), biotransformation of toxic compound in aquatic systems (Yang *et al*., 2011) and corrosion prevention (Videla and Herrera, 2005). Research therefore aims to discover synergistic combinations of species to improve productivity and function of biofilm communities.

Complex interactions occurring within community biofilms, including communication through quorum sensing molecules, suggests microorganisms are social entities completing sophisticated processes (Cremer *et al*., 2019). It is generally assumed that bacteria interactions within biofilms are a balance of cooperative and competitive (Elias and Banin, 2012). However, within many model systems differing results conclude either competition or cooperation dominates interactions in microbial communities (Foster and Bell, 2012; Poltak and Cooper, 2010).Competition can occur between residing species for limiting resources, known as resource competition, and bacteria can also exhibit interference competition through active secretion of toxins which kill neighbouring species (Friedman and Gore, 2016) This can result in a net decrease in biofilm community productivity (Tait and Sutherland, 2002), and effect the relative abundance of competing community members. Conversely, a cooperative or positive interaction can result in synergy, in which species share common resources, co-occur through different metabolic strategies or exhibit cross-feeding, which can display an increase in community biofilm productivity compared to monoculture (Poltak and Cooper, 2010).

Biofilm communities are thought to have a higher degree of interactions between cells because of extracellular polymeric substances (Xavier and Foster, 2007). The EPS is often termed ‘Public goods’, which are costly to produce, but many members within the community can benefit (West *et al*., 2006). However, this opens the community to cheating members that utilises secreted products without producing any (Boyle *et al*., 2013). The usage of secreted products such as quorum sensing molecules or iron-scavenging molecules by cheaters often results in instability, by decreasing community productivity or possible biofilm collapse (Griffin, West and Buckling, 2004; Popat *et al.,* 2012). Similarly, not all molecules produced by residing community members are beneficial, as secondary metabolites and metabolic waste products can be toxic to neighbouring species. Waste products and metabolites can affect the environmental pH within the biofilms, known as a ‘Public bad’, and the increase or decrease of environmental pH can negatively affect all community members (Ratzke, Jonas and Gore, 2018). Determining the importance and occurrence of interactions

within community biofilms is challenging, as the more complex the community, the harder it is to determine which species are interacting and how. However, these interactions shape biofilm function and properties, and knowledge from interaction studies can be applied to anti-biofilm therapeutics and improvement of ecologically beneficial biofilms.

Multi-species biofilms are in a constant state of change caused by small changes in the environment, interactions and through introduction and dispersal of community members. This changes community diversity and subsequent properties and functions within the biofilm. A key research interest is to understand changes within community biofilms overtime, through the observation of succession patterns (Røder, Sørensen and Burmølle, 2016). This can help understand or even predict the progression of acute biofilm infections or ecological important biofilm communities, but with the complexity of biofilm communities, this can be extremely challenging (Jackson, Churchill and Roden, 2001). Succession and selection studies are also relevant to industry, such as water distributions systems, to understand the dynamic of multi-species biofilms within to ensure safe delivery of water (Douterelo, Fish and Boxall, 2018). As condition in situ are hard to replicate in the laboratory, in vitro studies with model communities are commonly used. The internal and external environment can be changed to study a specific effect on selection, and a model community can provide more tractability. Although model systems are not specific to an environment, key ecological and evolutionary theory can be observed or tested. One conceptual model for succession of biofilm communities suggests species diversity is related to variation in resource diversity, competition and niche generation, and as the biofilm community ages interactions become an important factor in determining the structure and diversity within the community(Jackson, Churchill and Roden, 2001; Jackson, 2003). Model systems continue to discover new patterns of succession and development in microbial biofilms and provide a system to test existing ecological or evolutionary theory. Microbial model systems continue to be extremely important in biofilm research, and while many single-species model bacteria exist, several challenges exist when developing or choosing a multi-species model community.

Model communities are considered a closed assembly of microorganisms that represent or mimics the system behaviours of ecological communities under controlled conditions (Blasche *et al*., 2017). Model systems can range from a single-species or a synthetic dual-species community of known laboratory strains, to a natural community selected from the environment and inoculated straight into a laboratory experiment (Figure 1.2.3). However, with increased complexity comes a decrease in tractability, causing difficulty in analysis. It is

therefore important to choose the appropriate microbial community based on the research aims and objectives.

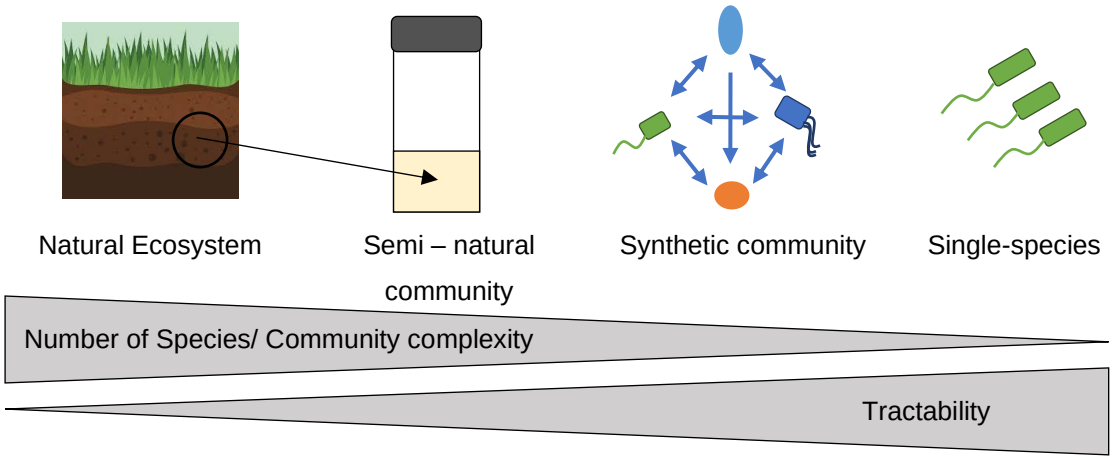


Figure 1.2.3. Model Communities for biofilm research. Model communities for biofilm research can different in complexity and tractability. Single or synthetically constructed systems are highly reproducible and easy to control by the experimenter but has very low ecological relevance to natural microbial communities. As the system increases in complexity the system begins to reflect the natural scenario, but this provides challenges in analysis, reproducibility and tractability. Figure modified from Blasche *et al*. (2017).

Synthetic communities can range in complexity from two to multiple strains, and still provides tractability as the experimenter can control the exact quantity and strains being inoculated into the system (Groβkopf and Soyer, 2014). Here, the direct interaction between two known species can be studied (Dubey and Ben-Yehuda, 2011; Christensen *et al*., 2002). However, this does still not reflect natural occurring ecosystems. To bring the system closer to the natural scenario clinical or environmental isolates can be used. Here the ecological and evolutionary dynamics within biofilm infections can be studied (Filkins *et al.,* 2015) and environmental isolates can provide an ecologically relevant model community (Burmølle *et al,* 2006). In environmental community samples such as soil it can be assumed that isolates coexist in nature and are more relevant to successional studies in multi-species biofilms than laboratory strains (Røder, Sørensen and Burmølle, 2016). These strains can be introduced into a biofilm experiment, building in complexity, measuring the impact on biofilm properties and biomass to determine cooperation or competition within (Ren *et al*., 2015; Roder *et al*., 2015). The most ecological relevant approach is the direct inoculation of environmental sample creating a semi-natural community (Blasche *et al*., 2017). Natural samples have

been taken from soil (Burmølle *et al*, 2006; Burmølle *et al*., 2007 Ren *et al*., 2015), streams or aquatic environments (Besemer *et al.,* 2007) and ponds or sands (Golby *et al*., 2012) to study ecological or evolutionary dynamics within biofilm communities. Samples can also be taken from a specific environment of interest such as food processing plants (Roder *et al*., 2015) and waste-water systems (Douterelo, Fish and Boxall, 2018).

There are still limitations to model communities, as model systems are still not fully representative of nature as it is extremely hard to replicate ecosystem function within a laboratory setting. However, the knowledge generated from model community biofilm studies can be used to predict or understand the dynamics of biofilm infections and improve the productivity and properties of industrial relevant biofilms for bioremediation or waste-water treatment (De Ro, *et al*., 2014; Song *et al*., 2014). Choosing a model community for the study of multi-species biofilms research is extremely important, with advantages and drawbacks (see Balsche *et al*., 2017), and this must be factored into the experimental design and research intentions.

1.2.4 Methods for Biofilm Study

Most of our knowledge of biofilms comes form in vitro model systems, which are easier to control than in situ studies (Røder, Sørensen and Burmølle, 2016). Model biofilm systems can be studied under flow or under static conditions. In flow systems a steady flow of nutrients is introduced, and waste products are removed, with flow cells (Pamp *et al*., 2009) or drip flow reactors (Goeres *et al*., 2009) often analysed directly with microscopy. Within flow cells solid-liquid biofilms can form on the solid surface and the biofilms matrix develops into the flow of nutrients and dissolved O_2 (Flemming and Wingender, 2010). Here the nutrients introduced can be easily controlled, and conditions can mimic the environmental conditions of biofilms forming in food processing equipment or water distribution systems where biofilms form on the inside of pipes and are subject to a constant liquid flow (Douterelo, Fish and Boxall, 2018). Biofilm studies can also be done under static conditions, where secondary metabolites and waste products can accumulate allowing further study of ecological dynamics such as cross-feeding, ecosystem engineering and niche-construction. Static systems include the use of microtiter plates, suitable for high-throughput analysis, where solid-liquid biofilms form in the well or on a coverslip added to the well (Harrison *et al*., 2010; Standar *et al*., 2010), or biofilms at the air-liquid interface form (Wiijman et al., 2006). Small glass vials, termed microcosms, are also used predominantly in static A-L interface biofilm studies. In microcosm vials 6ml of broth is typically added to create a large liquid column and biofilms can form at the air-liquid interface throughout attachment to the glass

vial wall (Rainey and Travisano, 1998). Biofilm characteristics can be measured using a combined biofilm assay (CBA, Robertson *et al*., 2013), combining measurement of cell density (OD_{600}) as an indicator for biofilm growth, cell growth, biomass or productivity, biofilm attachment (crystal violet staining, A_{570}) and biofilm strength using small glass beads (maximum-deformation mass, grams). Small glass beads can also be added to static biofilm systems, where solid-liquid biofilms can form, and beads can be removed for biofilm analysis (Poltak and Cooper, 2010). Biofilms can form in a diverse range of environments, and although the properties of each biofilm can be very specific to a single environmental within a laboratory setting (Sutherland, 2001), key concepts and properties of biofilm-formation and development can be captured. Despite the many biofilm experimental set-ups, few have been used in multi-species biofilm evolution experiments, and typically only consist of a two-species community (Hansen *et al*., 2007).

Within biofilm model systems simple enumeration assessment including cell density (OD_{600}) and plating for colony forming units (CFU) can show enhancement and inhibitory effects from evolutionary or ecological processes. Similarly, measuring changes in fitness, colony morphology, strain phenotype and behavioural traits are important measures in successional changes occurring in biofilms, as demonstrated in the *Escherichia coli* B long term evolution experiments (Lenski, 2017). However, the EPS makes up ~90% of biofilm mass and provides insight into the properties and structure of a biofilm (Flemming, Neu and Woznaik, 2007). New methods are being tested to further investigate the biofilm matrix (Sheng, Yu and Li, 2010). Initial tests using stains can indicate the type of polymeric substances being expressed by biofilm-forming strains. A common stain used in biofilm-based assays is crystal violet, which stains both the matrix and bacterial cells purple. This can be used to quantify biofilm attachment by eluting stain from a vial or plate wall with ethanol and measuring at 570 - 600nm (Spiers *et al*., 2002). Congo red, a common histology stain, can differentiate cells that can or cannot form biofilms using amyloid proteins, cellulose and glucan which are stained red, common biofilm matrix components. Other matrix component stains include calcofluor suggesting cellulose-like fibres are present within the matrix (Spiers *et al*., 2003), and DMMB (1,9dimethylmethylene blue) staining acidic polysaccharides (Toté *et al*., 2008). Staining can be viewed and imaged with microscopy, another diverse method for biofilm analysis.

Microscopy and imaging techniques allow analysis within the biofilm, and when coupled with staining or the addition of fluorescently tagged cells can show structure and composition within. Confocal laser scanning microscopy (CLSM) can view a biofilm in layers and coupled

within staining can identify key matrix substances and distribution of cells, and quantify marked strains of interest through GFP tagging (Lawrence *et al*., 1991; Neu and Lawrence, 2015; Tombolini and Jansson, 1998). Similarly, in-situ hybridization can view hydrated biofilm samples and does not require strains of interest to be fluorescently tagged (Amman and Fuchs, 2008). To view biofilm structure, scanning electron microscopy (SEM) has been developed to avoid the dehydrating step which would destroy the biofilm matrix, and biofilms can be fixed and covered with gold or platinum for analysis (Asahi *et al*., 2015). Atomic force microscopy can also be used to view the surface and three-dimensional structure of a biofilm (Defrêne, 2015). Microscopy and many other chromatography and spectroscopy approaches are used to study the composition and spatial distribution of biofilms, and extraction of EPS components for further identification and quantification (Sheng, Yu and Li, 2010).

Within evolutionary and ecological biofilm studies a key aim is to identify the species present and how the species diversity or species richness changes over the time. Similarly, an individual strain can demonstrate phenotypic changes, and therefore possible genetic changes in response to environment stressors or interaction with neighbouring species. This has been made easier with the advances in molecular and genetics technique over recent years. A common approach is the extraction of plasmid DNA and 16S rRNA (ribosomal ribonucleic acid) sequencing for taxonomic characterisation indicating species diversity (Besemer *et al*., 2012). This can be used to examine successional changes within community biofilms samples (Jackson, Churchill and Roden, 2001; Douterelo, Fish and Boxall, 2018). Similarly, DNA and qPCR analysis can provide more quantitative genetic analysis, assessing the individual ratio of bacteria species (Ren *et al*., 2014), indicating species richness within the community. However, limitations exist with some molecular approaches as some species may have the same 16s rRNA region and therefore produce the same band in denaturing gel electrophoresis (Jackson, Churchill and Roden, 2001). Further advances in metagenomics can identify taxonomic diversity within the community down to the strain-level classification (Tringe *et al*., 2005; Fierer, Baberán and Laughlin, 2014). Whole-genome sequences can be completed on individual strains of interest and can identify new strains or mutations within known strains. While genetic methods can provide information on the overall diversity and taxonomic make up of biofilm communities in successional studies, recent evidence suggests genomics and functional diversity are not necessarily mapped (Kraemer *et al*., 2010; Vos and Velicer, 2006). Rather, a single bacterial species can demonstrate many different functional traits and behaviours as a result of the environmental conditions or composition of the community. Further analysis of changes in

traits and behaviours are therefore important in assessing adaption or selection for specific community traits within biofilm studies.

1.2.5 Concluding Remarks

Bacterial biofilms are ubiquitous in nature, serving important ecological roles, and extremely problematic in clinical settings. A main aim of biofilm research is to development methods for the destruction or disruption of infectious bacterial communities, or the enhancement of ecological and biotechnological relevant biofilm communities. With biofilms, ecological and evolutionary dynamics shape the resulting properties of the biofilms, and this is influenced by environmental conditions and taxonomic make-up of the community. It is therefore important to understand patterns in the successional changes of bacterial biofilms to help understand the progression of acute infections, and ecological relevant biofilm communities. The experimental set-up for biofilm studies is extremely important, and the overall research questions and aims must be considered when selecting the appropriate model bacterial community and analysis techniques. Although model communities and experimental biofilm set-ups do not exactly replicate the condition in nature, they still prove invaluable insight into the ecological and evolutionary dynamics that shape biofilm communities.

1.3 Model Bacterium *Pseudomonas fluorescens* SBW25

1.3.1 An introduction to the *Pseudomonas* genus

Pseudomonas species, a Gamma-subclass of the Protobacteria, are a well-studied genera within microbiology. *Pseudomonas* spp. are found in a diverse range of habitats from soil and plant microbial communities, to human infections, reflected in the metabolic versatility found within the genus. Pseudomonads demonstrate a wide range of metabolic diversity including the ability to degrade a large range of organic compounds, including aromatic compounds (Stainer, Palleroni and Doudoroff, 1966) creating adaptability in changing environments (Silby *et al.*, 2011). Many Pseudomonads are opportunistic pathogens, causing disease in plants and human diseases, most notably the role of *P. aeruginosa* in cystic fibrosis infections. There is a broad range of potential biotechnological applications including bioremediation, biocontrol and plant growth promotion (Silby *et al*., 2001). Several revisions of the *Pseudomonas* genus have been made since the initial identification of *P. pyocyanae* in the late nineteenth century, now known as *P. aeruginosa.* The initial description of the genre was very basic, with Walter Migula of the Karlsruhe institute naming and describing the *Pseudomonas* genus as cells with polar organs of motility, with formation of spores rare (Migula, 1894 and 1900). They are now considered a gram-negative, rod-shaped and motile microorganism, unable to form spores (Palleroni, 2010) and are closely related to several other commonly recognised bacteria genera.

Seminal work determining diversity within *Pseudomonas spp* in 1966 characterised 267 strains (Stainer, Palleroni and Doudoroff, 1966), and although to date many of these strains may have moved class due to the introduction of DNA analysis. However, a large core group still remain and the *Pseudomonas* genus is still considered one of the most diverse range of species. Ribosomal RNA analysis was developed to define the taxonomic hierarchies of strains currently classified under the *Pseudomonas* genus, and five main rRNA groups were identified (Palleroni *et a*l., 1973). One group contained *P. aeruginosa,* fluorescent pseudomonads, and some non-fluorescent species such as *P. stutzeri,* and this rRNA group formed the '*Pseudmonas sensu stricto*', while the other four groups were re-classified to other genera's (Palleroni *et al*., 1973). There are now 191 recorded *Pseudomonas* species and this number continues to grow (Palleroni, 2008), and continuing DNA-DNA hybridisation (DDH), rRNA analysis and whole-genome sequencing allows for further resolution and definition of the *Pseudomonas* genus (Silby *et al*., 2011).

Pseudomonas cells are rod shaped, straight or slightly curved, with an approximate size of 0.5 -1.0 X 1.5 – 5.0 µm. The gram-negative cells are motile by polar singular or multiple flagella, and some species have fimbriae (pili). Many species including *P. aeruginosa* produce type-4 pili which are involved in cell adhesion, and twitching motility (Palleroni, 2015). Twitching motility is a form of surface translocation, where pili extend, attach to solid surfaces and retracts which pulls cells forward. Other sensing motility is also utilised by many species within the *Pseudomonas* genus, including chemical and oxygen sensing motility known as chemotaxis and aerotaxis (Taylor, Zhulin and Johnson, 1999). This motility is flagellum-dependent and can move cells along chemical or oxygen gradients. Aerotaxis is known to be important in air-liquid interface biofilm-formation, by positioning cells at the interface where O_2 concentration is high, and cells can subsequently attach and from biofilms (Hölscher et al., 2015). Although aerotaxis motility is thought to be common amongst aerobic bacteria and pseudomonads, and aerotaxis sensors have been annotated within the genome of *Pseudomonas* spp (Silby *et al*., 2009), little experimental verification of this motility is available.

Species within the *Pseudomonas* genus are metabolically and physiologically versatile, and as a result are found in a vast array of environment from human infections to terrestrial and aquatic environments (Palleroni, 1992). Most pseudomonads are aerobic, utilising O_2 as the final electron acceptor (Palleroni, 2015). However, some species including *P. aeruginosa* have been reported to utilise nitrate as an alternate electron acceptor and undergo anaerobic metabolism as a result of diversification and adaption within chronic cystic fibrosis infections (Hassett *et al.,* 2002). Denitrification is common amongst pseudomonads, with an estimated 50 genes involved in the reduction of nitrate to ammonia (Zumft and Körner 1997). *Pseudomonas* spp are capable of degrading a large variety of organic substrates, in particular aromatic compounds, through the production of oxygenase, and all species analysed to date undergo the tricarboxylic acid cycle (Krebs Cycle) (Palleroni, 2015). *Pseudomonas* spp are highly adaptable, and metabolically flexible as a result of allelic differences amongst common genes within the *Pseudomonas* genus, and many species such as *P. aeruginosa* and *P. syringae* have become sophisticated human and plant pathogens through horizontal gene transfer. Genome rearrangement likely contribute to adaption, and cause strain and species-specific activity (Silby *et al*., 2011).

Many *Pseudomonas spp* are opportunistic pathogens, most common *P. aeruginosa* which thrive in immunocompromised patients entering airways, urinary tract and open wounds, causing a range of septic and blood infections. *P. aeruginosa* thrives in damp conditions and

is often the fatal step in diseases such as cystic fibrosis, where thick *P. aeruginosa* biofilms form in mucus filled lungs, work which receives high focus in medical and clinical microbiology (Wagner and Iglewski, 2008). *P. aeruginosa* is one of the most studied *Pseudomonas* pathogens, and research suggests many interacting systems that work in response to environment stressors or cues controls virulence. This includes a two-component regulators system which controls virulence and resistance (Gooderham and Hancock, 2009), a quorum sensing system, the type III secretion system and sigma factors (Whitely, Lee and Greenberg, 1999; Potvin, Sanschagrin and Levesque 2008; Hauser, 2009). Biofilm-formation is common amongst *Pseudomonas* spp. with great diversity amongst the make-up of the EPS matrix between species. Key matrix component include extracellular DNA (Jahn, Griebe and Nielsen, 1999; Nivens *et al*., 2001) partially acetylated cellulose and PNAG in *P. fluorescent* strains (Spiers, 2014), polysaccharides alginate and Pel in *P. aeruginosa* strains (Yang *et al.,* 2011) and Levan and alginate in *P. syringae* strains (Laue *et al*., 2006).

Many *Pseudomonas* spp. cause infection in plants, including *P. putida* and *P. syringae,* and crop pathogens *P. corrugata, P. marginalis* and *P. viridiflava* (Hunter and Cigna, 1981; Kůdela, Krejzar and Pánková, 2010). Both human and plant infections from *Pseudomonas spp* continues to grow in severity with the rise in antimicrobial resistance and constant adaption and diversification within the species. However, pseudomonads also play an important ecological role. *P. fluorescens* P-5 and SBW25 promote health and nutrition in plants, forming on plant roots and leaves and contributing to the turnover or organic material in soil (Silby *et al*., 2009). The genus is ubiquitous in soil, and as a result have been used in the removal and detoxification of contaminated environments, known as bioremediation (Wasi, Tabrez and Ahmad, 2013). The strain *P. fluorescens* SBW25 has become a model bacterium in evolutionary and ecological studies (Koza *et al*., 2017), and advances in whole genome sequencing and molecular techniques, this strain can be readily manipulated. As a result, *P. fluorescens* SBW25 will be the model bacterium for the basis of this research.

1.3.2 Adaptive radiation of *Pseudomonas fluorescens* SBW25 in experimental microcosms

Pseudomonas fluorescens SBW25, isolated from sugar beet in Oxford UK (Bailey and Thompson, 1992), is a plant growth promoting pseudomonad, from the *P. fluorescens* species complex (Silby *et al*., 2009). Unlike the vast body of work on *P. aeruginosa, P. putida* and *P. syringae,* up until 2010, *P. fluorescens* strains received less attention within

research (Palleroni, 2010). *P. fluorescens* strains produce fluorescent pigments, some which have iron binding abilities (i.e., siderophores). Many fluorescent pseudomonads play key ecological roles within soil including *P. fluorescens* Pf0-1 and *P. protegens* Pf-5 (Silby *et al.,* 2011; Paulsen *et al.,* 2005) and colonise the phyllosphere by *P. fluorescens* SBW25 and A506 (Bailey and Thompson, 1992; Loper *et al*., 2012). However, although less virulent than *P. aeruginosa*, *P. fluorescens* is now recognised as a human pathogen, found in cystic fibrosis, acute pneumonia and transplant infections (Scales *et al*., 2014). The *P. fluorescens* species complex contains a diverse range of strains, with many functional traits and metabolic strategies. *P. fluorescens* SBW25 was initially studied for biotechnological application as an enhancer for plant growth and protection for crops against disease (Rainey and Bailey, 1996), however once statically incubated in liquid microcosm vials, its potential as a model system for the study of adaptive radiation and A-L interface biofilm-formation became apparent. The adaptive radiation of *P. fluorescens* SBW25 has been studied in 30ml glass vials containing 6ml of Kings B medium (Rainey and Travisano, 1998), and the diversification into morphologically-distinct biofilm-forming mutants is reproducible, occurring within approximately 100 generations spanning one to three days. This system can be incubated with shaking to provide a homogeneous environment, or statically where an O_2 gradient is established through metabolic activity, creating a heterogenous environment (Rainey and Travisano, 1998).

When static liquid microcosms are inoculated with wild-type *P. fluorescens* SBW25 cells, initial colonists deplete the liquid column of O_2, resulting in O_2 becoming a limited resource within the system (Koza *et al*., 2011). Random mutations result in the diversification of wild-type SBW25 into niche-specialists. Under standard conditions non-biofilm-forming wild-type SBW25 populations are quickly dominated by Wrinkly Spreader (WS) mutants capable of biofilm-formation at the air-liquid interface. Biofilm-formation allows WS mutants to access the high-O_2 region in static liquid microcosms, block the A-L interface from competitors and prevent further O_2 from diffusing into the liquid column where wild-type SBW25 cells remain (Koza *et al.,* 2011; Loudon *et al*., 2016). WS mutants therefore receive a competitive fitness advantage over the non-biofilm-forming ancestors, with increased access to O_2 and faster growth rates (Rainey and Travisano, 1998). The lower liquid column also supports populations of Fuzzy Spreaders mutant (FS mutants), thought to thrive under micro-aerobic conditions (Rainey and Travisano, 1998). Later experiments show FS mutants do attempt biofilm-formation at the A-L interface, but biofilm are too fragile and collapse to the bottom of the vial, suggesting poor biofilm-formation results in colonisation of the lower liquid column rather than adaption to low-O_2 condition (Ferguson, Bertels and Rainey, 2013). This system

provides a model for the study of adaptive radiation in bacteria and A-L interface biofilm-formation.

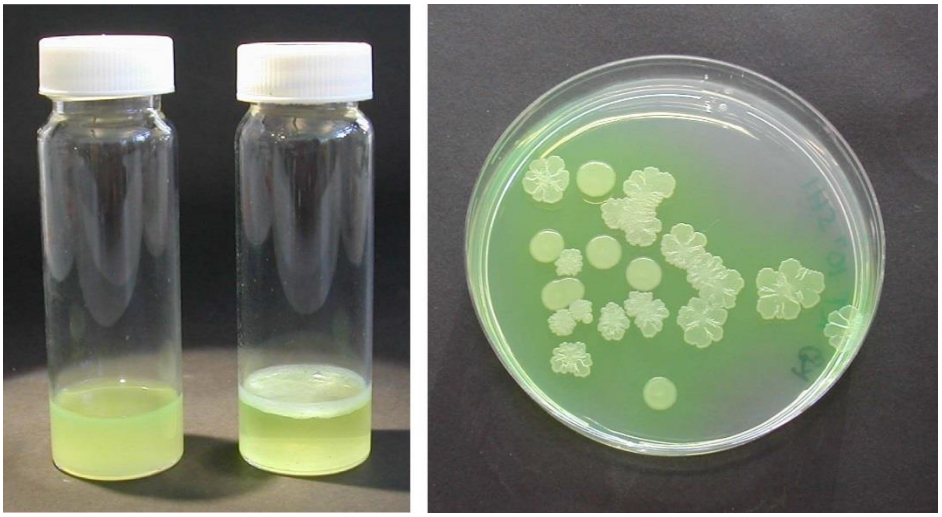


Figure 1.3.1 The diversification of *P. fluorescens* SBW25 leads to adaptive biofilm-forming mutants known as Wrinkly Spreaders. Wild-type SBW25 cannot form A-L interface biofilms, however random mutations lead to the rise of biofilm-forming mutants known as Wrinkly spreader. Wrinkly Spreaders can form A-L interface biofilm in static liquid microcosms (left) and can be differentiated on plates by a wrinkled and spreading colony morphology from the smooth colony morphology of wild-type SBW25. This Image is from Koza *et al*. (2017).

1.3.3 Underlying molecular biology of *P. fluorescens* SBW25 wrinkly spreader mutants

The underlying molecular biology of the WS mutant has been extensively investigated (reviewed by Spiers, 2014). The rise of the WS mutant phenotype within rapidly diversifying populations of *P. fluorescens* SBW25 is a result of mutations affecting cyclic-di-GMP homeostasis causing the over-production of cellulose, the key matrix component. The WS phenotype operon '*wsp*' (Figure 1.3.2) encodes the regulatory component required for the WS phenotype (Spiers *et al*., 2002; Bantanki *et al.,* 2007), and gene function is modelled on the highly-conserved Che Chemosensory system of *Escherichia coli* (Bren and Eisenbach, 2000). Within this 7 gene operon a membrane-associated receptor signalling complex containing methyl-accepting chemotaxis proteins (WspA), scaffold proteins (WspB and WspD) and histidine kinase (WspE) responds to external stimulus, possibly environmental stimulus or surface contact (based on the behaviour of the homologous *wsp* proteins in *P. aeruginosa* (Hickmann, Tifreama and Harwood, 2005; Güvener and Harwood, 2007)). This

activates the diguanylate cyclase (DGC) WspR response regulator, a regulatory protein also found in many other pseudomonas (Winsor *et al*., 2002). Activity with this complex is balanced by the opposing activities of the addition and removal of methyl groups (CH_3) by a methyltransferase (WspC) and methylesterase (WspF), which controls the activation of WspR in wild-type SBW25. Mutations occurring in WspF causes inhibition of methylesterase activity (Bantanki *et al*., 2007), or within WspE leading to over-activation of WspR inhibiting histidine kinase (McDonald *et al*.2009) result in the Wrinkly spreader phenotype. The enzymatic catalysis of GMP to cyclic-di-GMP by DGC WspR is activated by phosphorylation, and this cyclic-di-GMP activates the membrane-associated cellulose synthase biosynthetic responsible for the structure of WS mutant biofilm, known as the *wss* operon (WS Structural, Spiers *et al.,* 2002).

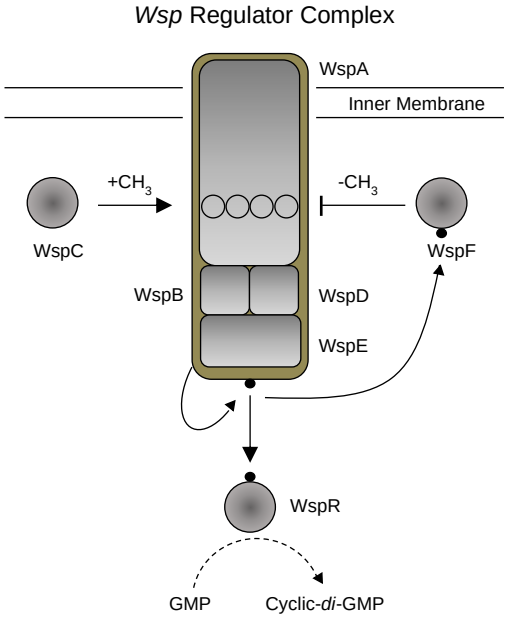


Figure 1.3.2 The Wrinkly spreader phenotype regulator operon *WsP*. The regulatory operon responsible for the WS mutant phenotype contains 7 genes including the methyl-accepting chemotaxis proteins (WspA), scaffold proteins (WspB and WspD) and histidine kinase (Wsp E). Activity of the regulatory complex (shown here as a schematic) is balanced by methyltransferase (WspC) and methylesterase (WspF), which prevents the activation of WspR, a DGC response regulator. Mutations have been found to occur in WspF or WspE which activates WspR leading to the production of cyclic-di-GMP. This activates the membrane-associated Cellulose synthase complex which results in the structure of the WS mutant. This figure was adapted from Spiers et al. (2014).

Within the *wss* operon, *wssB-E* share significant homology to the bacterial cellulose synthesizing operon, *bcs*, of *Gluconacetobacter hanasenii ATCC* 23769 (previously known as *Acetobacter xylinum)* and *E. coli* K-12 (Saxena *et al*., 1994; Blattner, *et al*., 1997). From

both DNA and protein-level homology WssB was identified as the cycli-di-GMP binding subunit, which catalyses the polymerisation of UDP-glucose to cellulose (Speirs et al., 2002). The *wss* operon contains additional genes WssA and WssF-J, in addition to the basic *bcs* operon. WssA and WssJ are thought to be responsible for the localisation of the cellulose synthase complex to the cell poles (Spiers *et al*., 2003) WssF – WssI, which share homology with the alginate acetylation proteins of *P. aeruginosa* FRDI (Franklin and Ohman, 1998) which are believed to be involved in the partial acetylation of cellulose. This partially-acetylated cellulose is then utilised as the major matrix component of the WS biofilm matrix, with thick cellulose fibres forming around voids and creating and extensive network throughout in which bacterial cells associate with (Spiers et al., 2002; Spiers et al., 2003). Cellulose expression is common within pseudomonads, with cellulose synthase homologues present in *P. putida* KT2440 and *P. syringae* DC300 (Nelson *et al*., 2002; Buell *et al.,* 2003; Ude *et al*., 2006), and many soil associated isolates have been shown to express cellulose (Ude *et al*., 2006).

In addition to mutations leading to the over-production of partially-acetylated cellulose, WS mutant cells also have an unidentified attachment factor (Spiers and Rainey, 2005). The fimbriae-like attachment factor is likely to be a poly-N-acetylglucosamine (PNAG) putative curli or thin aggregative fimbriae (Spiers *et al*., 2002; Spiers and Rainey, 2005; Lind, Farr and Rainey, 2015). Interactions between cells, cell debris and extracellular polymeric substances (EPS) including lipopolysaccharides (LPS) and cellulose fibres lead to the strength and integrity of the WS mutant biofilm (Spiers and Rainey, 2005; Lind, Farr and Rainey, 2017).

1.3.4 Microbial ecology of *P. fluorescens* SBW25 diversification

Initial wild-type SBW25 colonists act as ‘ecosystem engineers’ or ‘niche-creators’ within static liquid microcosms. Within the first three hours of static incubation wild-type SBW25 cells rapidly deplete the lower liquid column of O_2 through metabolic activity, resulting in a heterogeneous environment and creation of a new niche (Day, Laland and Odling-Smee, 2003; Steenackers *et al*., 2016). The influx of atmospheric O_2 into the system allow the upper liquid region to remain high in O_2, creating ecological opportunity within the system. A steep O_2 gradient is established, with only 0.1% the normal dissolved O_2 below 1 mm after five hours and less than 1% of the normal dissolved O_2 below the top 200 µm after five days (Koza *et al*., 2011). The low-O_2 liquid region takes up 90% of the liquid column within the microcosm (Koza *et al*., 2011). The rapid O_2 up-take can be described as a social dilemma known as the tragedy of the commons. O_2 within the system is a shared resource and is

depleted without restraint by selfish members and future generation have to survive a depleted environment, leading to the inevitable collapse of the population (Estrela *et al.*, 2019). However, the upper 200 µm region of the liquid column remains rich in O_2 and is often referred to as the 'Goldilocks zone' (Kuśmierska & Spiers 2016; Koza *et al.* 2017). Growth rates within this region are higher through increased O_2 access, making this region the optimal growth zone within the microcosm (Kuśmierska & Spiers 2016; Koza *et al.* 2017). This niche represents ecological opportunity for any adaptive lineage capable of colonising and exploiting it (Schluter, 2000; Yoder *et al*., 2010; Wellborn and Langerhans, 2015). The ability of biofilm-formation in WS mutants allow for colonisation of the 'Goldilocks zone', increasing growth rates and obtaining a competitive advantage by blocking other competitors from accessing the A-L interface and removing most O_2 from diffusing into the lower liquid region.

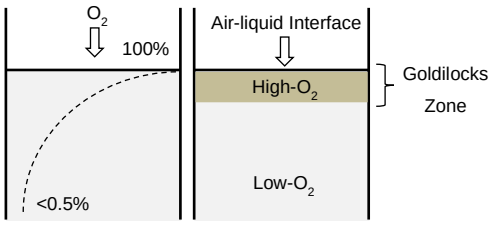


Figure 1.3.3 Initial wild-type SBW25 colonist establish O_2 gradient in static liquid microcosms. O_2 in the lower liquid region is depleted by initial wild-type SBW25 colonists creating an O_2 gradient within the first 5 hrs of incubation (left schematic). The upper liquid region directly below the A-L interface remains rich in O_2, and this region is known as the Goldilocks zone (right schematic). This figure was modified from Koza *et al*. (2017).

The O_2 gradient continues to intensify within the WS biofilm, with the top surface in contact with air remaining high in O_2, but within the biofilm O_2 continues to deplete (Koza *et al*., 2011). This is likely to produce further diversification within the lineage, evidenced by phenotypic and fitness variation found in WS mutants (McDonald *et al*., 2011; Lind, Farr and Rainey, 2015; Udall *et al.,* 2015), resulting in competition between WS mutants. Variation in WS mutant fitness and phenotype is characteristic of the 'Red Queen' hypothesis, a term used in evolutionary ecology based on The Red Queen character from Lewis Carol's book 'Through the looking glass", where in the Red Queen race she and Alice were constantly running yet remained in the same position (Liow, Van Valen and Stenseth, 2011). This hypothesis states that lineages must constantly evolve and adapt to compete within an everchanging environment. However, this does open the population to cheating phenotypes.

Within any cooperative system cheaters are likely to arise. Here, cheaters no longer produce cellulose contributing to biofilm-formation, while benefiting from the production of others (Rainey and Rainey, 2003; Brockhurst *et al*., 2006; Brockhurst *et al*., 2007). Cheaters strain the biofilm community, leading to instability and even biofilm collapse.

Liquid microcosms can also be incubated with shaking, in which no O_2 gradient is established. Shaking incubation ensures O_2 is constantly entering the system and does not become a limiting resource within nutrient rich KB microcosms. When WS mutants and wild-type SBW25 cells are re-introduced into a microcosm and incubating with shaking, the WS loses the competitive fitness as they are unable to form biofilms within this environment (Spiers *et al*., 2002). Similarly, on agar plates WS mutants are unstable, and can revert back to wild-type smooth colonies (Spiers, 2007). Diversification also occurs within shaking microcosms, with specialists arising within the population that can utilise different carbon sources (Mclean, Dickson and Bell, 2005).

Although O_2 availability is considered the dominant selective pressure in static microcosms, nutrient availability is also known to affect the diversification and fitness of WS mutants. Diversification has been examined with different media type present within static microcosms and the phenotype, wrinkeality (first used in Udall *et al.* 2015 to describe differences in colony morphology between WS isolates), biofilm characteristics and fitness of WS mutants arsing within the population is significantly altered (MacLaughlin, 2016). This suggests WS mutants have different metabolic effects which altered phenotype and fitness. Similarly, limiting nutrient availability by diluting KB media within the system also effects growth rate and biofilm characteristics is observed (Kassen, Llewellyn and Rainey, 2004, Koza *et al*., 2011, Kuśmierska and Spiers, 2016). It is clear that nutrient availability can also have a selective effect within the system, however naturally established nutrient gradients and the effect on diversification within the SBW25 system is less clear. Initial wild-type colonists are like to act as chemical ecosystem engineers through metabolic activity. The uptake of nutrients and production of toxic waste products and secondary metabolites can alter the chemical environment, including environmental pH which can have significant effect on residing cells within the system, including the engineering strain (McNally and Brown, 2015). The effect of this chemical environmental modification on population growth and the evolution of the WS mutant is unknown but would offer further insight into the ecological and evolutionary dynamics occurring with the *P. fluorescens* SBW25 system.

1.3.5 Other mutants within the *P. fluorescens* SBW25 lineage

The WS mutant has been extensively studied within the SBW25 system, however are not the only biofilm-forming mutant from the *P. fluorescens* SBW25 lineage. From populations of cellulose-deficient SBW25, two mutant strains were found to arise also capable of forming physical-cohesive class biofilms. Poly-acetyl glucosamine-Wrinkly Spreader (PWS) and Complementary Biofilm-forming Strain (CBFS) over-produce poly-acetyl glucosamine (PGA) as the main matrix component (Lind, Farr and Rainey, 2017; Gehrig, 2005). Both mutants demonstrate competitive fitness advantage over the wild-type SBW25 under standard conditions, and produce strong PC class biofilms, despite not producing cellulose which is required for the WS biofilms.

Although wild-type SBW25 is generally considered to be non-biofilm-forming, when induced with various heavy metals, in particular iron (III) chloride which induces cellulose production, SBW25 can form biofilms at the A-L interface, known as a viscous-mass (VM) biofilm. The VM biofilm matrix is composed of cellulose, however is weaker than the WS mutant biofilm likely due to a lack of attachment factor (Koza *et al*., 2009) As a result the VM biofilm can collapse with little physical disturbance, but like many other biofilm-forming mutants, also benefits from a fitness advantage compared to wild-type SBW25 (Koza *et al.*, 2009). The VM biofilm has a cloudy liquid column suggesting a substantial proportion of cells remain within the liquid column, unlike the WS mutants which has a transparent liquid column (observation, Spiers' research group). A cloudy liquid column is also found in CBFS mutant static cultures, but to a lesser extent than the VM biofilm. This suggests that it is not just the biofilm characteristics and EPS matrix that differs between adaptive mutants, but also the extent of A-L interface colonisation. Although adaptive mutants, partially-engineered mutants and induced biofilms within the *P. fluorescens* SBW25 have not evolved directly from the same lineage, they have all adapted or convergently evolved to A-L interface biofilm-formation in static liquid microcosms. Further differences between these A-L interface biofilm-forming mutants could be explored to determine if some adaptive mutants are more suited to biofilm colonisation than others through fitness comparisons, and if biofilm characteristics or increased cell localisation to the A-L interface provide a fitness advantage within this microcosms system.

1.3.6 Future Work for the *P. fluorescens* SBW25 system

While the *P. fluorescens* SBW25 system has a substantial body of work that spans almost 30 years, there are still some knowledge gaps that remain within the literature. Colonising

the high-O_2 region is key to successful growth for *P. fluorescens* SBW25 within this system, however is A-L interface biofilm-formation the only strategy capable of doing this? Biofilm-formation is a costly strategy, with energy expenditure need for attachment and production of the extracellular matrix. Within this system WS mutants require energy for the polymerisation of UDP glucose to produce cellulose, the key matrix component (Spiers *et al*., 2002). However, if cells could position themselves within the high-O_2 region without biofilm-formation, this could enhance fitness, as energy otherwise used for biofilm-formation could be utilised for population growth, suggesting a resource trade-off. O_2 sensing motility, known as aerotaxis is shown to move cells along an O_2 gradient, which may be a key non-biofilm-forming colonisation strategy to colonise the high-O_2 region in static liquid microcosms. *P. fluorescens* SBW25 is thought to be aerotaxis (Silby *et al*., 2009), and if functional within wild-type SBW25 cells could act as a non-biofilm-forming colonising strategy. This questions the need for biofilm-formation at the A-L interface, if a more cost-effect motility strategy could position cells within the high-O_2 region. Further research is needed to determine the fundamental need for A-L interface biofilm formation by determining why this costly strategy provides a fitness benefit compared to the alternate aerotaxis-mediated colonisation.

A clear difference between the WS mutant biofilm, and the VM biofilm formed by wild-type SBW25 when induced with iron is the visual appearance of the top surface of the A-L interface biofilms. WS mutant biofilm are dry-looking, while the VM biofilm is wet and fragile (Koza *et al*., 2009). This suggests a difference in association with the A-L interface, indicating WS mutant cells have an additional mechanism to break through the interface for biofilm-formation to occur above the interface in contact with air. However, the interface between air and liquid is difficult to break, due to surface tension created by strong hydrogen bonding between water molecules. Bacteria cells must interact with the interface to inhabit this region, through the production of organic molecules which would lower surface tension (Preston and Roger, 2005). This allows cells to lower ST and break through the A-L interface, position cells above the interface to begin biofilm development. The production of cellulose, lipopolysaccharide (LPS) and a possible attachment factor contribute to the WS mutant ability to form biofilms at the A-L interface. However, WS mutant cells need to access and interact with the A-L interface for biofilm-formation to occur above the A-L interface. This suggests and additional step or mechanisms is required to break through the A-L interface for successful and robust biofilm-formation to occur.

The research gaps highlighted within the *P. fluorescens* SBW25 system will be explored throughout this research, where I hope to investigate the need and mechanisms of A-L interface biofilm-formation in static liquid microcosms.

1.4 Thesis Aims and Objectives

1.4.1 Study Rational

I identified key knowledge gaps in our understanding of why A-L interface biofilm-formation occurs. Although it is clear that the WS mutant has a fitness advantage over the non-biofilm-forming wild-type SBW25, it still remains unclear why the biofilm strategy is more successful than other non-biofilm-forming colonising strategies. This will be investigated within the *Pseudomonas fluorescens* SBW25 system, by first confirming aerotaxis in *P. fluorescen*s SBW25 cells. A comparison of the relative success of the biofilm and aerotaxis colonising strategies can then be made, comparing localisation of cells to the high O_2, involving the development of a quantitative assay to measure cells distribution throughout the liquid column. To determine how WS mutant cells successfully interact with the A-L interface surface tension analysis will be used. Finally, the fitness benefits of biofilm-formation and cell localisation characteristics will be compared with the WS mutant and other mutants within the SBW25 lineage. This will allow a broader consideration of the relative importance of A-L interface biofilm-formation and cell localisation to the high-O_2 region for strains competing in static microcosms. Stronger biofilm-formation is often considered a measure of success or fitness but does not take into consideration how strains use the entire environment, where the liquid column can support growth despite low-O_2 conditions. Here, I aim to further develop the current model of A-L interface biofilm-formation in the SBW25 system to include the fundamental need and relative success for biofilm-formation.

While single-species systems answer key questions surrounding fundamental aspects of biofilm-formation and development, they do not represent the natural scenario. Static liquid microcosms provide a system where the dominant selective pressure is limiting O_2. O_2 gradient are ubiquitous in nature, suggesting the microcosm models system can be further developed to form an ecologically relevant multi-species biofilm system. This will require the selection and validation of an appropriate model community, with high diversity and relevance to A-L interface biofilm-formation. Using a serial-transfer approach to represent pulse disturbance or re-seeding events, short term evolution experiments can be designed, where evolutionary changes have been observed over short time periods. Within community ecology there has been a recent focus in understanding community changes based on functional and trait-based changes, and how individual-level traits influence emergent community properties. This research will therefore focus on changes in community productivity, biofilm-associated and phenotypic based changes between the initial bacteria community and community after selection. Here, the key eco-evolutionary dynamics which

shape the succession of bacterial strains within a serially transferred community in response to an O_2 gradient can be investigate, including interactions between species and the environment. Although model system do not mimic a specific ecological or hospital based environment, the development of multi-species model systems is still vital to understand key evolutionary and ecological dynamics within microbial communities. A multi-species microcosms system will represent a complex community, of interacting and competing species, where dynamics of A-L interface biofilm-formation can be investigated at a community and individual strain-level, and changes in productivity can be understood.

It is clear that O_2 is a limiting resource within static liquid microcosms, and the establishment of O_2 gradients creates a significant selective pressure. However, nutrient availability is also likely to have a selective effect, through chemical ecosystem engineering. Metabolic activity withing growing systems is inevitable, and the uptake of nutrients and production of secondary metabolites and waste products will likely alter the chemical composition of the surrounding environment. Even small changes in environmental conditions can have a profound effect of microbial populations, which can inhibit growth. Chemical ecosystem engineering will be explored by returning to the SBW25 system, where the effects of chemical ecosystem engineering by initial wild-type SBW25 colonists can be explored. Chemical changes within the media and how this effects the key aspects and processes within the diversification of *P. fluorescens* SBW25 including growth, evolution, biofilm characteristics and fitness will be investigated. Ecosystem engineering is of growing interest in microbial ecology, and the effects of inevitable processes such as metabolism can further our understanding of how microbial populations can change over short time periods, by exploring the relationship between microbial process and the environment. This will also enhance our understanding of ecological dynamics within static liquid microcosms.

1.4.2 Aims and Objectives

Chapter 3: Understanding air-liquid interface biofilm-formation using the *Pseudomonas fluorescens* SBW25 model system

1. Confirm the swimming motility of wild-type *P. fluorescens* SBW25 and the Wrinkly Spreader mutant and provide experimental proof of aerotaxis in these strains.
2. Compare the relative value of biofilm-formation and aerotaxis strategies for the colonisation of the high-O_2 region of static liquid microcosms.
3. Investigate A-L interface–cell interactions for wild-type SBW25 and the Wrinkly Spreader during the early development of biofilms.

4. Explore the link between fitness benefits of biofilm-formation and cell localisation characteristics of Complementary biofilm-forming strain (CBFS), Viscous mass (VM) and Wrinkly Spreader cells.

Chapter 4: Development of a microcosm model system to investigate selective effect of O_2 limitations on community-aggregated traits and productivity in multi-species air-liquid interface biofilm-forming communities

1. Develop a microcosm system to study selection within multi-species biofilm-forming communities.
2. Explore the effect of selection in heterogenous O2 limiting conditions on community-aggregated and individual strain-levels straits of A-L interface biofilm-forming communities.
3. Investigate the influence and interaction between coalescing biofilm and non-biofilm space.
4. Explore the resistance of selected communities to invading species. .

Chapter 5: Understanding chemical ecosystem engineering in the *Pseudomonas fluorescens* SBW25 model system.

1. Create aged media with wild-type SBW25 cells and provide evidence of ecosystem engineering by chemical changes.
2. Explore the effect of the modified environment on the evolution and fitness of the WS mutant.
3. Determine any inhibitory effects of aged media on wild-type SBW25 and WS mutant populations

Chapter 2: Materials and Methods

All chemicals and reagents used throughout this research came from Thermo Fisher Scientific, UK, Sigma Chemicals, UK or Scharlu, Scharlab, UK.

2.1 Growth media

Kings B* (KB*) (King *et al*., 1954) broth used throughout this research was made with 1.5 g magnesium sulphate ($MgSO_4$), 1.5 g dipotassium phosphate (K_2HPO_4, 20 g Protease peptone No.3 and 10g glycerol per litre of de-ionised water. Microcosms were 30ml glass vials containing 6 ml of broth (Rainey and Travisano, 1998) with lids left loose and taped with micropore ape to ensure O_2 could enter the vial. For standard agar plates 1.5 % (w/v) agar was added to media to make 20ml plates. All media was autoclaved at 121°C for 30 minutes. Modified microcosms to prevent the induction of the VM biofilm produced by wild-type SBW25 were made with the addition of 20 µM 2,2-dipyridyl and 0.1 µM Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) (KB*-DP/T) (Koza *et al*., 2009).

Luria-Bertani media was made with 10 g Tryptone, 5 g Yeast extract, 10 g sodium chloride (NaCl) per L de-ionised water (Sambrook et al., 1989) and M9 minimal media with 1 x M9 salts (2 ml of 1M magnesium sulphate ($MgSO_4$), 100 µl of 1M calcium chloride ($CaCl_2$)) per L de-ionised water (Miller, 1972). *Pseudomonas* selective agar (48.2 g *Pseudomonas* selective base with L de-ionised water (Kielwein, 1969)) was used with a CFC supplement (cetrimide, fusidin and cephaloridine supplement powder dissolved in a 1:1 ethanol: water).

2.1.1 Additives and Antibiotics

Kanamycin (Km) was used at a concentration of 25 or 50 µg ml^{-1}, and filter sterilised into media using a 0.2 µm GD/X filter (Whatman, UK). X-Gal (BCIG, 5-bromo-4-chloro- 3-indolyl-beta-D-galacto-pyranoside) was dissolved in dimethyl formamide (DMF) and used at concentration of 40 µg ml^{-1} in plates. To visualise the oxygen gradient in static liquid microcosms, methylene blue was added to a concentration of 0.0005 % (w/v) and pictured after 24 h incubation.

2.1.2 Viscosity additives

Agar and polyethylene glycol (PEG 10000, Sigma) were added to standard KB* microcosms to increase the viscosity of media. Low, medium and high concentrations of agar (0.01, 0.05,

0.1 % (w/v)) and PEG (1, 2.5 and 5 % (w/v)) were selected to give a viscosity range of between 1.44 – 7.13 mPa s (work carried out by A. Kuśmierska and M. Petric, Spiers' research group), which is low enough to maintain media in a fluid state. Microcosms were heated briefly in the microwave and cooled to around 50˚C before inoculation.

2.1.3 Aged media

Aged media (spent media with prolonged growth period allowing for nutrient depletion, and waste product and metabolite accumulation) was created by incubating wild-type SBW25 or community samples in KB* broth, in shaking or static conditions, for 1 – 10 days. Cells were removed by centrifugation for 30 minutes at maximum speed in a 5810R Eppendorf centrifuge (maximum g-force 20800xg), and the supernatant was combined to make 6 ml microcosms. Microcosms with aged media were then sterilised by autoclaving to prevent any further growth.

2.2 Culturing conditions

All strains or community samples were stored cryogenically at -80˚C containing 700 µl of overnight culture and 300 µl of 50 % (w/v) glycerol solution. Prior to experimentation frozen stocks were re-streaked onto plates and incubated for 2 days to insure growth and contaminant free stock. For all experiments overnight cultures were created by inoculating fresh microcosms with frozen stock using a sterile 10 µl wire loop and incubated overnight with shacking in an automatic Stuart S150 orbital incubator at 150 rpm. Overnight samples of soil-wash inoculum were made using 100 µl of frozen soil-wash stock in microcosms. 100 µl of overnight cultures were used for starting experiments.

All *P. fluorescens* strains were incubated at 28°C and all soil-wash community and isolate samples at 20°C. With shaken incubation all lids were left loose and lightly taped (micropore tape) to ensure they stayed secure when shaking but were sufficiently aerated. For static growth conditions, microcosms were placed in a Laminar flow cabinet with loose lids at laboratory temperature (~20 - 23°C).

2.2.1 Growth assessment

For growth experiments where enumeration was required, bacterial cultures were serially diluted in sterile de-ionised water from 10^{-1} to 10^{-9} and 100 µl aliquots were spread onto plates. KB* plates were used for obtaining colony development and growth, and plates were

incubated for 2 - 3 days until colony development was visible and colony forming unit (CFU) counts could be made. Cell density or productivity of samples was measured using optical density at 600 nm (OD_{600}), using a Spectronic Helios Epsilon Spectrophotomer (Thermo Fisher Scientific, UK), with non-UV cuvettes with a 10 mm optical path.

2.2.2 Low-O_2 growth conditions

Growth in micro-aerobic conditions were performed using 2 L anaerobic jars containing Oxois AnaeroGen 2.5 L sachets to removes O_2, lowering the concentration to below 1 % normal O_2 levels within 30 minutes. Microcosm vials were placed in anaerobic jars after inoculation and were incubated statically with lids loose.

2.3 Bacteria and plasmids

2.3.1 Bacterial strains and plasmids

Bacteria strains and plasmids used throughout this research are listed in Table 2.1.

Table 2.1. Bacteria strains and plasmids used in this research

Strain	Genotype / Description	Source
***Pseudomonas fluorescens* SBW25**	SBW25 wild-type	Rainey and Bailey 1996.
FleQ$^-$	SBW25 *ΔfleQ*; flagella-deficiant mutant not capable of swimming	Alsohim *et al.*, 2014.
CheA$^-$	SBW25 *cheA::aph* (Kan^R); chemotaxis-defective mutant still capable of swimming	Spiers and Rainey, 2005.
Wrinkly Spreader (WS)	SBW25 *wspF* A901C; archetypal biofilm-forming mutant	Rainey and Travisano, 1998; Bantinaki *et al*., 2007
CBFS	SBW25 SG70 plus unidentified biofilm-activating mutation (Kan^R); Complementary biofilm-forming strain 2.1	Gherig 2005.
SBW25-*lacZ*	SBW25 *lacZ*$^+$; neutral marker	Zhang and Rainey, 2007.
WS-*lacZ*	SBW25 WS *lacZ*$^+$; constructed from SBW25-*lacZ* with pAS286 but now Kan^S, neutral marker	This Research.
SG70	SBW25 *Δwss;* cellulose-biosynthesis deficient mutant unable to form a biofilm, (Kan^R) *aph*^{3}	Gherig, 2005.
Plasmid	**Genetic Description**	**Source**
pAS285	Wild-type SBW25 *wspF* cassette carried by pCR201 vector (Kan^R)	Bantinaki *et al* 2007

pAS286	Wrinkly spreader *wspF* S01R mutation carried by pCR201 vector (Kan^R)	Bantinaki *et al* 2007

2.3.2 Soil-wash bacterial community and isolates

Soil was obtained from an allotment in Dundee in October 2017. A 200 g sample was added to 300 ml of sterile de-ionised water and mixed for 24 hrs at 125 rpm to break up the soil and produce a suspension. An additional 600 ml of sterile de-ionised water was added and mixed for a further 30 minutes. This soil suspension was aliquoted into 50 ml centrifuge tubes and centrifuged at 4,000 rpm for 25 minutes to pellet any solid material. The pelleted material was collected and resuspended in 7 ml of KB* broth to stimulate microbial growth and left for 1 hr. The contents of each tube were combined to produce an overall volume of 100 ml, and 100 ml of 50 % (w/v) glycerol solution was added in preparation for storage. The soil-wash solution was then divided into 300 µl aliquots stored in 1.5 ml centrifuge tubes and frozen at -80°C. After 14 days of storage at -80°C and overnight soil culture was prepared by inoculating 100 µl of the soil-wash sample into KB* microcosms. The overnight culture was serially diluted in replicate (n=4) and plated on KB* agar, and plates were stored at 22˚C for 2 days. Colonies were selected with a wire loop using a random stratified sampling approach. Four Colonies were selected from plates 10^{-5} to 10^{-7} from each replicate sample and re-streaked on fresh KB* plates and incubated for 2 days. Colony material was selected to prepare overnight cultures which was utilised to create -80˚C stocks.

2.3.3 Strain construction

2.3.3.1 DNA isolation

Plasmids were carried by *Escherichia coli* K12 general laboratory strains. Plasmid DNA was obtained from 5ml of overnight culture and extracted using a low-copy-number QIAprep spin miniprep kit (Qiagen) and stored at -20˚C. DNA was dialysed against 20ml of molecular grade deionised water using 0.025 µm cellulose membrane filters (Millipore, UK) for 1 hr.

2.3.3.2 Electrophoresis

Electrophoresis was used to confirm isolation of plasmid DNA. Gel red (Biotium, UK) was added to 1.5 % (w/v) Agarose-TBE gels for a final concentration of 1 $mg.ml^{-1}$. DNA samples were diluted to give a concentration range of 0.5, 0.2 and 0.1x, and were mixed with 0.1 volume of loading buffer (Fermentas, UK) before placing into the well. The gel was run for 1 -

2 hr at 100 V and visualised under UV-light transilluminator (Alphaimager, GRI, UK). A 1kb DNA ladder (Fermentas, UK) was used as a size marker.

2.3.3.3 Electroporation

Overnight cultures of the recipient strain were grown in KB* and 5 ml of cells were washed and re-suspended in 1 ml of cold 10 % (v/v) glycerol / 1 mM HEPES solution (Millipore, UK) to make electrocompetent cells. A 100 µl sample of electrocompetent cells was mixed with 5 µl of dialysed DNA and transferred to pre-chilled 0.2 mm GenePulser cuvettes (Bio-Rad, UK) and electroporated at 200Ω, 1.75kV, 25Mf using an Eppendorf electroporator (Eppendorf, UK). Electroporated cells were transferred to KB* microcosms and incubated with shaking for 90 minutes at 28˚C. Colonies were spread onto 25 $\mu g.ml^{-1}$ Kanamycin and 40 $\mu g.ml^{-1}$ X-gal 40 $\mu g.ml^{-1}$ KB* plates, and colonies were selected after 2 days of incubation.

2.3.3.4 Strain identification

A selection of soil isolates were identified by mass spectrometry with Biomérieux Vitek Moltitov Mass Spectrometry. This analysis was carried out by *Clinical Microbiology Identification* (Kiev, Ukraine). Samples were prepared using small amounts of colony material with 1 µl of lysing buffer provided by *Clinical Microbiology Identification*. Protein profiles from each isolate were compared to the Biomérieux VITEK MS V3 data base for species identification (Biomérieux, SA Ltd).

2.4 Microscopy

2.4.1 Standard Microscopy

Standard microscopy was viewed using a Leica DMR microscope, and images captured with a Sony EXWAVE HD 3CCD colour camera. Images were taken at 1000x magnification with immersion oil (Cargille, US).

2.4.2 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy analysis used a CTR Advanced Leica, Confocal Laser Scanning Microscope using LASX imaging software to obtain both 2-D and 3-D images. This analysis was carried out at the Institute of Molecular Biology and Genetics, National Academy of Sciences, Ukraine (Kiev). Samples were prepared by selecting biofilm material with a wire loop and placing on to a glass slide with sterile water, with the top surface of the

biofilm in contact with the glass slide. Samples were lightly fixed by fuming for 20 minutes with paraformaldehyde and multiple stains were added before adding a cover slip. All images were taken at magnification 63x. Proteins were stained with thiazine red (1 mg ml^{-1} Sigma-Aldrich), cells were strained green with Syber green (1 mg ml^{-1} Sigma-Aldrich) or DNA was stained red with ethidium bromide (1 mg ml^{-1} Sigma-Aldrich), cellulose-like polymers were stained blue with calcofluor fluorescent brightener 28 (1 mg ml^{-1} Sigma-Aldrich), extracellular DNA and metabolically inactive cells were stained red with eDNA 1276 (1 mg ml^{-1} Sigma-Aldrich) and amyloids were stained red using Amyloid 155 (1 mg ml^{-1} Sigma-Aldrich). Blue stains were viewed with a laser at 405 nm, red at 532 nm and green at 488 nm.

2.4.3 Scanning Electron Microscopy

A Joel JSM 35C Scanning Electron Microscope (SEM) was used to obtain images of single and mixed species biofilms at the Institute of Botany Genetics, National Academy of Sciences, Ukraine (Kiev). Double-sided tape was applied to each brass cylinder table and biofilm samples were placed onto the tape to ensure adhesion of biofilm material to the table. Samples were frozen using liquid nitrogen and placed in a compressed dryer for 5 hrs. Samples were then covered with gold layer of 15 – 20 Å before analysis.

2.5 Motility

2.5.1 Swimming motility

Soft-agar plater were used to assess swimming, containing 40 ml of 0.3 % (w/v) agar and 0.1x KB* nutrients which were left to set overnight to ensure the semi-solid agar had solidified. Replicate inoculations (n=8) of 10 µl were made through the agar. Plates were incubated upright and the diameter of swimming was measured in mm after 24 and 48 h.

2.5.2 Aerotaxis

2.5.2.1 Qualitative aerotaxis assay

To visualise aerotaxis motility by cells, a semi-solid test tube approach was developed. Glass test tubes (20 ml) were filled with 5 ml of semi-solid agar (0.3 % w/v) and left overnight to dry. A cell pellet was created by taking 500 µl of overnight culture and re-suspended in semi-solid agar and Tetrazoluim violet (2,5-diphenyl-3-2-napthyl tetrazoluim chloride, TV)

was added to give a final concentration of 20 µg.ml^{-1}. TV produces a purple colour in response to metabolic activity. The cell pellet was placed on top of the pre-set agar column and left to dry for 1 – 2 h A further 5 ml semi-solid agar plug was placed onto the cell pellet and left to dry before placing in the incubator at 28˚C. Qualitative results were recorded over a 72 h time period by observing the direction of cell movement from the cell pellet. Cell localisation at the top of the agar column in contact with air was recorded as a positive result for aerotaxis.

2.5.2.2 Quantitative aerotaxis assay

To quantify aerotaixs motility cell movement was quantified by counting cells at the A-L interface on microscope slides, with and without the presence of oxygen. A sample of overnight culture was placed onto a microscope slides, with three sides of the coverslip sealed with entellan. This created a small chamber, with one side still open to air. Slides were placed into a glass beaker and semi-sealed, and a steady stream of N_2 gas entered the system for 30 minutes. Slides were quickly removed and visualised under the microscope with pictures captures at the interface between the culture and air at 1000x magnification. The slides were left for a further 30 minutes within a standard oxygen environment and further pictures were taken at the same location. This was repeated with replicates (n = 5). From pictures taken bacterial cells were counted within an established area at the edge of A-L interface, and an O_2/N_2 ratio was calculated. A ratio above one suggests cells were actively moving towards the A-L interface in the presence of O_2 suggesting aerotaxis motility.

2.6 Cell density

Cell density was calculated with replicates (n=5) by taking 50 ml samples of overnight culture and centrifuging maximum speed for 30 minutes to obtain a large cell pellet. The cell pellet was re-suspended in 500 µl of KB* and transferred to a pre-weighed 1.5 ml Eppendorf tube. After centrifuging for a further three minutes the supernatant was removed and the tube containing the cell pellet was weighed to obtain the mass of the cell pellet. Small aliquots of media were added to the pellet until 1 ml was reached to obtain the volume of the pellet, and the cell pellet density was calculated.

2.7 Surface tension

Surface tension (ST, mN m^{-1}) was measured using a K100 Mk2 tensiometer (Krüss, Germany) using a SV23 AI/PTFE conical sample vessel and platinum testing rod following

the established method (Fletcher and Pringle, 1985). Replicates (n = 3) of re-suspended cell samples, cultures and cell-free culture supernatants were measured at 20 °C. Deionised water and sterile KB-DP/T broth were used as controls and under these condition the ST measurements were 73.1 ± 0.18 mN $m^{-1}$and 43.6 ± 1.6 mN m^{-1}, respectively.

2.8 Cell localisation

To quantify cell localisation throughout the liquid column and cell enrichment to the high O_2 region in static liquid microcosms, cell density was measured for every 1 ml of the liquid column. Cell pellets were created by re-suspending 500 µl of overnight culture in 100 µl of fresh KB* medium. Replicate inoculations (n = 5) of 20 µl of the cell pellet was placed at the bottom of the liquid column of KB* microcosms, and were statistically analysed after 24 or 72 h. Sequential sampling from the top downwards of 1 ml was measured for cell density (OD_{600}), ensuring all biofilm material was collected within the first 1 ml sample. For each 1 ml sample, the relative cell density was calculated (OD_{600} at depth divided by total mean OD_{600}) to show cell distribution throughout the liquid column. A comparison of cell enrichment at the top 1 ml, and of the proportion of cells remaining in the liquid column (2^{nd} sample – 5^{th} sample) were made between samples.

2.8.1 Cell localisation is viscous microcosms

The cell localisation method was modified to measure cell enrichment into the liquid column in microcosms modified with viscosity agents. Viscosity agents and concentrations used were 0.01% (w/v) and 0.1% (w/v) technical Agar, 1% (w/v) and 5% (w/v) Polyethylene glycol (PEG 10,000; Sigma). After static incubation a 1 ml sample was taken from the bottom of the liquid column and OD_{600} measured, and the remainder of the culture was vortexed vigorously. A 1 ml sample for the remaining mixed culture was measured and data is shown a cell distribution in the liquid column (mixed/ bottom OD_{600} ratio) where a ratio less than one suggests fewer cells have migrated into the liquid column from the inoculation point at the bottom of the liquid column.

2.9 Biofilm characterisation

The combined biofilm assay (CBA) was taken from Robertson *et al*. (2013) to quantitatively measure biofilm strength (maximum deformation mass, grams), total growth (optical density, OD_{600}) and biofilm attachment using crystal violet staining (Sigma basic violet 3, 0.05 % w/v). Maximum deformation mass was measured using small glass balls of uniform weight (mean 0.115 g) which were placed onto the biofilm until the biofilm broke or sank (Spiers *et al*,

2002). The content of each microcosm was transferred to fresh microcosm, vortexed vigorously and 1 ml was taken to measure OD_{600}. The remaining empty vials were washed to remove any unattached cells and 1 ml of crystal violet added to each vial. Using racks to contain the vials, a 3 dimensional figure of eight motion for two minutes provided sufficient movement to ensure the crystal violet could stain any attached cells. The remaining crystal violet was washed out and 200 µl of ethanol was carefully placed at the bottom of each vial and removed to ensure any staining of the vial not related to biofilm attachment was removed. The remaining attached stain was eluted with 5ml of ethanol and shaking for 2 hours. Samples were measured at 570 nm (A_{570}) to give levels of attachment in each sample (Spiers *et al*, 2003).

2.10 Phenotypic and behavioural characterisation

2.10.1 pH indication

Bromocresol green solution (0.025 g powder with 25 µl de-ionised water, BDH Laboratory supplies) was added to 50 µl of overnight cultures and left for 10 seconds before results recorded. For a positive result a colour change from green to blue occurs, implicating cultures have a pH above 3.8 to 5.4. Any other colour changes were recorded as a negative result.

2.10.2 Catalase production

To test for catalase production 5 µl of hydrogen peroxide (H_2O_2) was placed in 20 µl of an overnight culture. Positives results were recorded when samples produced bubbles immediately after the addition of hydrogen peroxide, indicating the decomposition of hydrogen peroxide to water and O_2 through the enzyme catalase. Samples with no immediate production of bubbles were recorded as negative.

2.10.3 Oxidase production

The oxidase test was used to determine if isolates produce cytochrome c oxidases suggesting the ability of cells to utilise O_2 as an electron acceptor, by converting O_2 to H_2O_2 or H_20 with an electron transfer chain. 5 µl of N,N,N′,N′-tetramethyl-p-phenylenediamine (Sigma Chemicals, UK) was placed onto a 20 µl drop of overnight culture on a petri dish lid and left for 10 seconds. Positive results for oxidase production were recorded when culture

samples turned blue at 10 seconds. Any samples that remained colourless or changed blue after 10 seconds were recoded negative for oxidase production.

2.10.4 Surfactant production

To test for the production of surfactant-like or surface-active agent molecules a drop collapse assay (Jain *et al*., 1991) and foam production assay (Cooper and Goldenberg, 1987) was used. A 50 µl drop of overnight culture was placed on to a petri dish next to a 50 µl drop of sterile water as a control. Any culture that remained as a droplet, like the water was recorded as a negative result. Positive results were recorded when the droplet collapsed suggesting surfactants were being produced. Similarly, the production of stable foam after vigorous shaking was also used to indicate the production of surfactants. Overnight cultures for individual strains were shaken for two minutes and the height of foam was recorded in millimetres, where a measurement of over 1cm of foam were recorded as positive result for surfactants in this research.

2.10.5 Siderophore production

To test for siderophore production overnight cultures were plated on KB* and incubated for 2 days. Any presence of fluorescent yellow colour within and surrounding the colony was recorded as positive for siderophore production.

2.10.6 *Pseudomonas* selective growth

Isolates were also grown on *Pseudomonas* selective agar plates with a CFC supplement, which are selective for growth of pseudomonads. Isolates capable of growing on PSA-CFC plates were recorded as positive, suggesting isolate may be a *Pseudomonas* spp.

2.10.7 Gram Staining

To categorise isolates into gram positive and gram negative a standard gram staining protocol was followed. Samples of colony material on KB* plates were utilised for staining. Samples were fixed onto microscope slides using heat and sterile water. Slides were flooded with crystal violet for 60 seconds followed by Lugol's Iodine (1 g Iodine, 2 g potassium iodide and 100 ml sterile de-ionised water) for 60 seconds. Acetone was applied for 5 -10 seconds and quickly washed off, with the final addition of Safranin (1 g in 400 ml sterile de-ionised water) for 30 seconds. Safranin was washed off the microscope slide, and slides were viewed with microscopy at 1000x magnification.

2.11 Community Selection in microcosms

To demonstrate a selective effect in bacteria community microcosms, community productivity (OD_{600}) was compared when incubation type (shaken or static), incubation period (days) and media type were compared. A decrease in community productivity in response to changes in incubation conditions suggested a selective effect on the community. The initial soil-wash community was grown over-night and inoculated into KB*, LB and M9 with glucose microcosms with replicates (n=16). One set of replicates (n = 4) were set in laminar flow cabinets and grown statically, and the other set were placed in an orbital shaker. On each sampling day 50 µl was removed from each sample and OD_{600} was measured. Sampling took place after 1, 3, 6, 10 and 13 days of incubation. Results were used to design the community serial transfer selection experiment.

2.11.1 Community serial-transfer experiment

The initial soil-wash community was used to inoculate KB* microcosms for the starting community for the serial-transfer experiment which contained nine treatments. Replicate communities (n=3) were set for each treatment, within replicate microcosms (n=8) set for each community. At each transfer the first seven replicate microcosms were destructively sampled using the combined biofilm assay (Robertson *et al.,* 2013), and the eighth microcosm was transferred to create the next replicate samples in fresh KB* microcosms. The replicate communities were transferred ten times across eleven microcosms, in which sample transfer type (biofilm or mixed community) incubation type (static or shaken) and period of incubation between transfers (one, three and six days) varied to make up nine transfer experiments. For mixed community transfers 100 µl of the final replicate in each community was transferred to eight new microcosms after vigorous mixing. Within the biofilm-only transfer regime a wire loop was used to select a small section of biofilm material and was mixed with 1 ml of sterile water, and 100 µl was used to inoculate the next set of microcosms. At the end of ten transfers each replicate community sample was serially diluted and plated on KB* in replicate (n = 3). Twelve colonies were selected over three plates in each serial dilution (four colonies from 10^{-5} to 10^{-7}) using a stratified random sampling approach. Colonies were selected to reflect community diversity and richness, based on size, texture and colour, and additional colonies were samples for dominant colony morphologies found on plates.

2.11.2 Assessing changes in community diversity after serial-transfer

To determine changes in diversity as a result of serial-transfer, 24 isolates from the initial soil-wash community and 24 isolates from the final transfer community (n = 8 per replicate community) were chosen for further analysis. The biofilm characteristics of each isolate was measured with the combined biofilm assay and a series of phenotypic and behavioural assays. The entire biofilm assay data set for all isolates was combined and split into four using median and quartile ranges. From this each isolate was given a 3-digit code (e.g., 342 – medium strength, high growth, low attachment). Similarly, this was completed for the phenotype data set, where positive and negative results were converted to a numeric value (positive = 1 and negative = 2), giving each isolate an 8-digit phenotype code. Each unique code was used to represent an operational taxonomic unit (OTU) which allowed species indices for each community to be calculated. The Simpson's Reciprocal Index (D) and Shannon Index (H) (Stirling, 2002) were chosen to calculate species indices.

Simpson Reciprocal Index:

$$D = 1 / [\Sigma n(n-1) / N(N-1)]$$

Where:

- n = Total number of organisms of a particular species.
- N = Total number of organic of all species.

D value begins at 1, representing a diversity of only 1 species, and as D increases so does the diversity of the community.

Shannon Index:

$$H = [\Sigma pi \, In \, pi]$$

Where:

- Pi = Proportion of indices found in the *i*th species in the data of interest

H values are usually between 1.5 and 3.5, where as H increase so does the number of species within the community, affected by both species' diversity and species richness.

2.12 Evolution Assays

Evolution assays were carried out so assess the percentage of WS mutants arising in populations of wild-type SBW25. Microcosms containing KB* broth or aged media were inoculated with 100µl of overnight culture and incubated statically for three days (unless otherwise stated). Samples were serially diluted and plates were incubated at 28˚C for two days. CFU counts were made for wild-type SBW25 and WS mutant colonies, and calculated to give the percentage of WS mutants arisen in the population. Where required, WS mutant colonies were selected and stored at -80˚C for future experiments.

2.13 Fitness Assays

Competitive fitness (W) was explored in pairwise competition assays to giver the change in frequency of two strains grown together. The competitive fitness of SBW25 strains relative to each other, or communities were performed in KB* replicate microcosms (n=5). In standard fitness assays a 1:1 mixture was made of competing strains or communities, and 100 µl used for starting inoculations. When testing 'invading from rare' a 1:1000 ratio was used, where one represented a rare phenotype arising in an established community. Initial CFU counts for each strain or community was used to adjust the actual starting ratio. After three days of static incubation cultures were diluted and plated and CFU's were counted for each strain or community. Where differentiation between colony morphologies could be made, no marked strains were used. When competitive fitness assays consisted of an invading SBW25 strain within a community, Lac-Z constructed strains were utilised on KB* X-gal plates. A competitive fitness (W) greater than one suggests that strain has a competitive advantage over the other, termed an adaptive or beneficial genotype. Competitive fitness (W) was calculated using the ratio of Malthusian parameters (Lenski *et al*., 1991):

Malthusian parameters:

$$W = ln [T_f / T_i] / ln [R_f / R_i]$$

Where:

- *ln* = Natural log
- T = Test strain
- R = Reference strain or community
- f = final
- i = Initial

2.14 Interaction Assays

2.14.1 Combinational isolate analysis

To assess synergistic, competitive or neutral interactions between community member, productivity (OD_{600}) was compared between combinations of community isolates of varying diversity and the corresponding community sample. Isolates from selected community replicates from the serial transfer experiments were grown in combinations of one, two, four and eight isolates, and compared to the entire community, with the total inoculum size always equal to 100 µl. Competitive interactions would be indicated by a decrease in community productivity compared to community isolates. A positive correlation between the

number of community isolates and productivity would suggest synergistic interactions and no changes would suggest neutral interactions.

12.14.2 Spot-on-lawn interaction Assays

Antagonistic and competitive interactions were tested using a “spot-and-lawn” method (Schillinger and Lucke, 1989). Overnight cultures of the lawn strains were diluted 10^{-3} before spreading onto plates, and leaving to dry for 1 hr. The strain of interest was spotted on top of the lawn strain, with 10 µl used for each spot. If normal growth occurred for both strains results were recorded as a neutral interaction. Where growth from the lawn strain was lighter where the two strains came in to contact a competitive interaction results was recoded. Finally, if a clear inhibition zone around the spot strain was observed, an antagonistic interaction was recorded.

2.15 Chemical Analysis

2.15.1 dsDNA Quantification

Double stranded DNA was quantified in aged media samples using a NanoDrop one Microvolume UV-Vis spectrometer (Thermo Fisher Scientific). The Nanodrop was blanked using sterile de-ionised water, and 1 µl of aged media was used for analysis.

2.15.2 pH

The pH of aged media samples was measured with an Elutech benchtop pH 510 probe (Cyberscan, UK) at room temperature. The probe was placed in replicate microcosm (n = 3) containing 6 ml of aged media samples.

2.15.3 Fourier-transform Infrared Spectroscopy

FTIR spectra of KB* AND aged media was collected using a Nicolet is5 FTIR, with atr attachment (Thermo Fisher Scientific, UK). Transmittance (%) was recorded for each wavenumber cm^{-1} between 400.16 cm^{-1} and 4000.12 cm^{-1}. Prior to sample analysis background noise was collected which was eliminated from sample profiles automatically.

2.16 Images

Images were take using an IPhone SE camera. Additional images used were provided by A. Spiers.

2.16 Statistical analysis and modelling

Experiments were conducted with replicates (n = 3 - 8). Means and standard errors are shown when data was normally distributed, and boxplots or median plus range is shown when data or residuals are not from the normal distribution. All statistical analysis, unless otherwise stated, was performed using JMP Statistical Discovery Software 12 (SAS institute inc. USA). All statistical analysis and modelling, including those presented in our papers, was conducted collaboratively with Dr Spiers. I prepared data sheets, undertook data quality checks including transcription errors from experimental notes, and produced exploratory figures as the key experimenter. These were then used in discussions to determine our approach to the formal statistical analysis which was undertaken with further discussion of the JMP output and results. During the campus closure when in-person meetings were not possible, Dr Spiers undertook the analyses remotely after discussion, and then provided the JMP output for my inspection and review. Final statistical and modelling outputs were agreed and JMP records were maintained as part of my PhD logbook. During the peer review of our manuscripts, we undertook further analyses of some data to accommodate reviewer's comments that small samples sizes and pseudo-replication issues might confound analyses. We undertook additional non-parametric tests to address the concerns regarding small sample sizes, and re-analysed data using general (linear) mixed-effect models to address the pseudo-replication concerns. These additional analyses are presented after my original statistical analyses in the relevant research chapters.

Data sets and residuals were tested using the Shapiro-Wilk W test to check for goodness-of-fit in the test for normality ($P > 0.05$), and to decide whether a parametric or non-parametric approach was appropriate. When data was from the normal distribution, parametric tests were used to test differences between means using t-tests ($P < 0.05$) or one-way analysis of variance (ANOVA) with Student's t or Tukey-Kramer honest significant difference (TK-HSD) *post hoc* tests ($\alpha = 0.05$) to show where significant differences occurred, and one-sample t-tests were used to compare means against a control mean. When data was not from the normal distribution, the Kruskall-Wallis (rank sum) test using the Wilcoxon method ($P < 0.05$) was used to compare differences between means and the Dunnett's method for Joint Ranking was used when comparing means with a control. The Dunns method of Joint

Ranking was used for tests with small samples sizes following reviewer's recommendation, to confirm results from the parametric tests.

General Linear (Mixed) Models (GLM/GLMM) were used when a modelling approach was needed for more complex data sets to identify which variables had the most influence in the experiment. General Linear Models were used to identify these variables in normally distributed data. General Linear (Mixed effects) Models were used with larger community data sets to identify key factors impacting changes in serial-transfer experiments (Chapter 4). Upon preliminary analysis and advice from reviewers, GLMM was used to overcome possible pseudo-replication issues, as the model allows microcosm replicate and community replicates to be nested. In addition, these variables could also be added as random effects, to account for the random variability found between replicate communities due to the stochastic nature of some evolutionary and ecological processes. Some GLMMs also contained multiple continuous variables. A Standard Least Squares approach and the Restricted Maximum Likelihood method with unbounded variance components were used with all GLMMs, and effects were investigated using LSMeans Differences Student's t and Tukey HSD tests ($\alpha = 0.05$). Non-normality was accepted in GLMMS, but no loss in degree of freedom and high *RSquared* values were required to produce robust models. The results of these models was further confirmed by GLMMs undertaken after the analysis of residuals and removal of outliers, without reducing the degrees of freedom or causing singularities, and using the Anderson-Darling test to confirm normality (using JMP version 15).

Means were further analysed using Hierarchical cluster analysis (HCA) using the Ward method giving equal weight for all factors and Principle Component Analysis (PCA) of correlations with Barlett's test of Eigenvalues to summarise patters and changes in data. To identify associations between means within experiments, Chi-Squared test of independence (socscistatistics.com), Spearman's ρ correlations ($P < 0.05$) and Odds ratios ($P < 0.05$) (www.medcalc.org) were used. Graphs were produced in JMP or Excel (Microsoft Office, 2020) and were re-dawn using PowerPoint (Microsoft Office, 2020) for manuscripts and this Thesis. Data is presented as mean ± standard error to two decimal places, and P values are recorded to three decimal places.

Chapter 3. Understanding air-liquid interface biofilm-formation using the *Pseudomonas fluorescens* SBW25 model system

Abstract

Static microcosms are a well-established system used to study the diversification of Pseudomonas fluorescens SBW25 and the adaptive A-L interface biofilm-forming mutants known as Wrinkly Spreaders (WS). WS mutants have a significant competitive fitness advantage over non-biofilm forming competitors and are better able to access the high-O_2 region directly below the interface of static microcosms through biofilm-formation. The underlying molecular biology and evolutionary ecology of the WS mutant is well known, however a key question remains unanswered: why is A-L interface biofilm-formation a more successful strategy than flagella-mediated aerotaxis as a means of colonising the high-O_2 region? Here, I show that wild-type SBW25 cells are capable of aerotaxis motility as expected for an aerobic flagellated bacterium. Through quantitative cell localisation experiments, I show that the WS mutant can achieve significantly higher cell densities in the high-O_2 region compared to wild-type and flagella-deficient mutant cells. Although aerotaxis allows wild-type SBW25 cells to move into the high-O_2 region from the lower liquid column effectively, they cannot counter the effects of physical displacement, through Brownian motion, vibrations and bioconvection or thermal currents, as efficiently as WS mutant cells which are also aerotaxic but also have an additional capability. WS mutant cells appear to better interact with the A-L interface than wild-type cells, with a surface-active-agent that lowered interfacial tension allowing the penetration of the A-L interface and subsequent development of a physically strong and well-attached biofilm. However, although A-L interface biofilm-formation provides the WS mutant with a fitness advantage over non-biofilm-forming competitors, other biofilm-forming mutants of the SBW25 lineage are able to out-compete the WS mutant. The induced viscous mass (VM) biofilm formed by wild-type

cells and the complementary biofilm-forming strain (CBFS) produce physically weaker biofilms than the WS mutant but maximise productivity by colonising the A-L interface. By colonising the remaining high-O_2 region immediately below the A-L interface, as well as the low-O_2 liquid column, the VM biofilm-forming wild-type cells and CBFS can achieve higher cell numbers throughout the whole system. This explains the fitness benefit they have over the WS mutant (fitness is determined for the entire microcosm and not simply for the A-L interface or high-O_2 region). The research presented here expands our understanding of A-L interface biofilm-formation using the SBW25 model system. It demonstrates that strong biofilm-formation does not always relate to success and that maximising productivity by colonising multiple niches throughout the static microcosm can improve fitness more effectively than just by colonising the A-L interface.

3.1 Introduction

The adaptive radiation of the model bacterium *Pseudomonas fluorescens* SBW25 into biofilm-forming mutants including the Wrinkly Spreaders (WS) has been explored through experimental evolution studies (see section 1.3). The molecular biology underlying biofilm-formation, and the ecological advantage of colonising the air-liquid (A-L) interface is clear within this system (Spiers, 2014 and Koza *et al.,* 2017). However, further research is needed to establish the fundamental need or value of biofilm-formation in this experimental system and to determine the key mechanisms used by cells to access and remain at the A-L interface.

The fitness advantage A-L interface biofilm-formation provides WS mutants over the non-biofilm forming ancestor has been clearly demonstrated (Rainey and Travisano, 1998, reviewed by Spiers, 2014). Many other biofilm-forming mutants have been found within the *P. fluorescens* SBW25 lineage, which differ in biofilm characteristics and matrix composition. Complementary biofilm-forming strain (CBFS) form physically cohesive biofilms without cellulose (Gehrig, 2005), and the wild-type SBW25 form weakly attached Viscous mass (VM) biofilms when induced with exogenous iron (Koza *et al*., 2009). Despite differences, the WS mutant and other SBW25 biofilm-forming mutants all possess a fitness advantage over the non-biofilm forming wild-type SBW25 (Spiers *et al*., 2002; Gehrig, 2005; Koza *et al*., 2009; Green *et al*., 2001; Lind, Farr and Rainey, 2017). There are many advantages to colonisation through biofilm-formation, with the extracellular matrix providing protection to cells against predation, physical displacement, chemicals, heavy metal, and antibiotics in a variety of environments and contexts (Flemming and Wingender, 2010). However, biofilm-formation is a costly colonising strategy. During biofilm development there is a resource trade-off between nutrients and energy needed for attachment and biofilm development and energy that could be utilised for cell replication and motility (Bachman *et al*., 2017; Arcerenza, 2016). Biofilm-formation at the A-L interface requires cells to be localised at the interface for attachment and biofilm development to be initiated. Cells positioned randomly throughout the liquid column must migrate upwards to the high-O_2 region. I postulate that aerotaxis (Taylor, Zhulin and Johnson, 1999), O_2 directed swimming motility, is important in the initial steps of positioning cells at the A-L interface within this microcosm system.

Motility is an important trait amongst bacteria and common amongst pseudomonads, however, published research characterising motility for specific strains is limited and has focused on the molecular mechanisms controlling this behaviour in model *Escherichia coli*

and *Bacillus subtilis* strains (Szurmant and Ordal, 2004; Berg, 2003). *P. fluorescens* SBW25 is known to swim several times faster than many peritrichous bacteria and other pseudomonads, utilising a monotrichous right-handed flagellum (Ping, Birkenbeil and Monajembashi, 2013). A combination of forward and backward swimming, with swift turns and flips to reorient the cell body and direction of motility, and the ability to "hover" in place, results in a sophisticated swimming pattern (Ping, Birkenbeil and Monajembashi, 2013). *P. fluorescens* SBW25 is also known to display energy-sensing motility such as chemotaxis (de Weert *et al*., 2002), and is suspected to be aerotaxic.

Aerobic and motile bacteria are generally assumed to be aerotaxic though this is rarely proven experimentally. The movement up an increasing O_2 gradient to the A-L interface is shown to give a competitive fitness over non-aerotaxic strains in *Bacillus subtilis* NCIB3610 and *Pseudomonas solancearum* BI-A (Hölscher *et al*., 2015; Kelman and Hruschka, 1973). Experimental verification of aerotaxis in *Pseudomonas* spp. is limited, however, *P. fluorescens* SBW25 contains an aerotaxis sensor annotated within the genome suggesting this stain is capable of aerotaxis motility(Silby *et al*., 2009). Clear experimental evidence of aerotaxis in *P. fluorescens* SBW25 cells would suggest that cells are capable of colonising the A-L interface without the need for biofilm-formation. If functional, *P. fluorescens* SBW25 cells may be capable of localising to the high-O_2 'Goldilocks' region in a cost-effective manner. This then raises the question of why is A-L interface biofilm-formation utilised by WS mutants, if aerotaxis is sufficient in locating cells within the high-O_2 region? By developing a simple assay to demonstrate aerotaxis in wild-type SBW25, I can make a comparison of aerotaxis and biofilm-formation can be made. Both strategies are known to localise cells to the high-O_2 A-L interface, but the relative success of each strategy in positioning and maintaining cells at the A-L interface has not yet been compared.

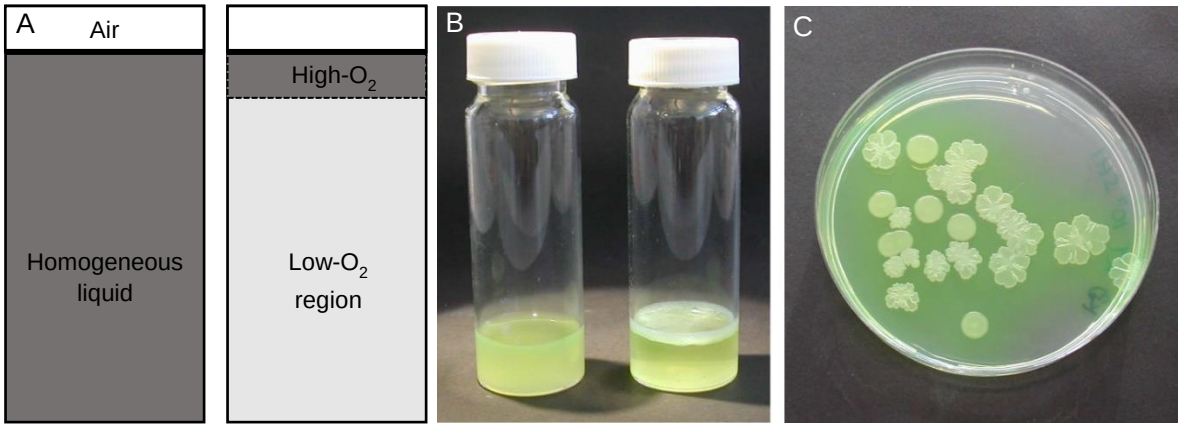


Figure 3.1. *Pseudomonas fluorescens* SBW25 model system. Ecological and evolutionary studies utilise microcosms to capture ecological dynamics within the evolving systems. Static liquid microcosms allow for the development of A-L interface biofilms. When inoculated with wild-type SBW25, the initial colonists act as ecosystem engineers depleting the lower liquid region of O_2 and creating a high-O_2 ecological niche directly below the A-L interface (A). Ancestral SBW25 is unable to form a biofilm in this environment, however random mutation leads to the rise of Wrinkly Spreaders and other mutants, capable of A-L interface biofilm-formation (B) and colonies can be differentiated on plates as wild-type SBW25 colonies are smooth and round and the WS mutants colonies are wrinkled (C). (Spiers, 2014).

To further understand the need for biofilm formation in static liquid microcosms, the abiotic environment must also be considered (Knopa, 2009). The chemical and physical environment can alter movement and behaviour of microorganisms, and similarly the movement of microorganisms can enhance these effects. Within the liquid column cells require upward directional swimming to achieve localisation at the A-L interface, and maintain position in this region to obtain the fitness advantage provided by increased O_2 access. Displacement caused by Brownian motion, bioconvection and thermal currents, and physical disturbance can re-position cells within the liquid column. Brownian motion, the random movement of particles suspended in liquid, influences bacterial swimming by displacing cells and randomizing the direction of both single and peritrichously flagellated bacteria (Berg, 1993, Guangali *et al*., 2008). In response to an attractant such as O_2 the frequency of ‘tumbles’ or ‘turns’ decreases, however Brownian motion can further randomise and rotate the running path causing inefficiencies (Mitchel and Kogure, 2005). Bioconvection currents (Hill and Pedley, 2005), caused by the upward swimming of microorganisms and subsequent displacement downwards due to a higher cell density than the liquid suspension (between 5-15% denser (Wager, 1911, Pedley and Kessler, 1992)), are also known to alter

the physical environment. The accumulation of cells at the A-L interface becomes unstable through increased density, and cells fall down. The combination of upward swimming and microorganisms falling down the liquid suspension creates bioconvection. Bioconvection currents have been reported to displace nutrients and oxygen into the liquid column, but recent evidence suggests this force is not significant enough for measurable difference in O_2 and nutrient concentrations (Janosi *et al*., 2002), and the ecological relevance of bioconvection has been questioned due to the specificity of conditions needed. It is unclear what the combined effects of Brownian motion, bioconvection currents and physical displacement has on colonisation at the A-L interface by wild-type SBW25 and WS mutant cells, with the microcosm model system.

Recent research (A. Kuśmierska, M. Petric; Spiers' research group) explored the effect of increased viscosity on fitness, resulting in a reduction of WS mutant competitive fitness comapred to wild-type SBW25 in static microcosms in which the liquid viscosity had been increased. Agar and polyethylene glycol (PEG) act as viscosity agents increasing intermolecular friction and networks (Armisen and Galatas, 2009; Zhao, Dimova and Liu, 2015), therefore reducing the physical displacement by Brownian motion, vibrations and thermal or bioconvection currents (referred to collectively here as physical displacement). Low to high concentrations of agar and PEG increasing viscosity (1-7 mPa s^{-1}) achieves this while keeping media fluid (i.e. without resulting in a visually obvious viscous mass or gel). Understanding the mechanism for the loss in WS mutant competitive fitness within a high viscosity environment needs further investigation. I hypothesise that increasing viscosity within this system reduces the impact of physical displacement, and motile cells are better able to remain in position at the A-L interface against disruption. The ability to swim would allow a greater number off wild-type SBW25 cells to localise and maintain position within high-O_2 region, increasing competitive fitness. Development of a quantitative cell localisation assay would allow me to compare comparison of cell localisation to the high-O_2 region with and without increased viscosity to determine the impact of physical cell displacement on A-L interface colonisation.

Increased cellulose and attachment factor expressed by WS mutant cells describes the structural nature of the WS mutant biofilm. The VM biofilm produced by wild-type SBW25 cells (induced by exogenous iron (Spiers *et al.,* 2006; Koza *et al*., 2009)) also uses cellulose and poly-acetyl glucosamine (PGA) extracellular matrix (Koza *et al*., 2009; Lind, Farr and Rainey, 2017), but distinct visual differences to the WS mutant biofilm suggests there are differences in how both biofilms are formed. The upper surface of the WS mutant biofilm is

dry, different from the wet and poorly attached VM biofilm (Table 3.1). Previous schematics of the microcosm model system show a three niche environment, consisting of the low-O_2 liquid column, the high-O_2 region at the top of the liquid and the interface between liquid and air where biofilm-formation occurs (Koza *et al*., 2017) (Figure 3.1, A).

Interfaces between air and liquid are difficult to break, and access by microbial cells require additional organic molecules to lower the interfacial tension (commonly referred to as surface tension) and break the nanometre-deep molecular layer (Kjelleberg, 1985; Berg, 2009). Within the study of interfaces, specifically the ocean-air interface often thought of as one giant gelatinous biofilm, complex layering of biotic and abiotic zones occur (Kjelleberg, 1985). Seminal work on the ocean-air interface uses the term ‘neuston’ to describe organisms associated with the water surface. The neuston layer has two distinct layers, the ‘epineuston’ composed of living organisms on the surface exposed to air, and the ‘hyponeuston’ to describe organisms positioned on the underside of the surface submerged in water (Marshall and Burchardt, 2005). Both are associated and interact with the interface, but in a different manner. Water surfaces containing organic matter and microbial communities are now considered as having two sides: the ‘air-side’ and the ‘water-side’ (Preston and Roger, 2005). Tensiometer analysis allows for the measurement of surface tension (γ), in which a platinum ring or rod is submerged, and the force required to pull out of the liquid is recorded. This technique has been utilised to determine the surface active properties of bacteria and the lowering of surface tension at both A-L and L-S interfaces (Rühs *et al.,* 2014; Mohammed *et al*., 2015). In particular, the role of cellulose and viscosin in lowering surface tensions in the VM biofilm produced by wild-type SWBW25 has been established (Koza *et al.,* 2009). Using this method, I predict a difference in surface-acting properties of wild-type SBW25 and WS mutants, which would provide evidence for the apparent differences seen between the two biofilm types and provide and mechanistic explanation for the ability of WS mutant biofilms to be located above the A-L interface.

Table 3.1. Comparison of A-L interface biofilm produced by wild-type *Pseudomonas fluorescens* SBW25 and mutants.

	Viscous Mass (VM)	**Wrinkly Spreader (WS)**	**Complementary biofilm-forming strains (CBFS)**
Biofilm Characteristics	Viscous mass, Low strength and weakly attached.	Physically cohesive, robust and well attached.	Physically cohesive, robust and well attached.
Biofilm Appearance	Wet and smooth 	Dry and rough. 	Dry and smooth. 
EPS/ matrix	PGA and low levels of cellulose.	PGA and high levels of cellulose.	PGA.
Liquid Column	Turbid, high growth.	Clear, low growth.	Intermediate, some growth.
Colony morphology	Smooth and round.	Wrinkled, rough and flat.	Small, round and rough.

3.1.1 Chapter Research Aims

The aim of this chapter is to further explore A-L interface biofilm-formation by the adaptive Wrinkly Spreader mutant and determine the physical reasons underlying the choice of biofilm-formation over aerotaxis motility for colonising the A-L interface in static liquid microcosms. This will require demonstrating aerotaxis by *P. fluorescen*s SBW25 cells and the development of a quantitative assay to measure cell distributions through the liquid column, after which a comparison of the relative success of the biofilm and aerotaxis strategies in localising and maintaining cells at the A-L interface can be made. Surface tension measurements will also be used to investigate how wild-type SBW25 and WS mutant cells access the A-L interface. Finally, a comparison of the fitness benefits of biofilm-formation and cell localisation characteristics of CBFS, VM and WS mutant cells will be made to allow a broader consideration of the relative importance of A-L interface biofilm-

formation and cell localisation to the high-O_2 region for strains competing in static microcosms.

3.1.2 Research Objectives

1. Confirm the swimming motility of wild-type *P. fluorescens* SBW25 and the Wrinkly Spreader mutant and provide experimental proof of aerotaxis in these strains.
    I. Compare the swimming motility and chemotaxis of wild-type SBW25 and the Wrinkly Spreader using semi-soft agar plate assays.
    II. Develop a quantitative microscopy-based aerotaxis assay and characterise the aerotaxis behaviour of wild-type SBW25 and the Wrinkly Spreader.

2. Compare the relative value of biofilm-formation and aerotaxis strategies for the colonisation of the high-O_2 region of static liquid microcosms.
    I. Develop a quantitative assay to determine cell distributions through the liquid column and characterise wild-type SBW25 and Wrinkly Spreader cell localisation to the high-O_2 region.
    II. Explore the impact of physical displacement on maintenance of wild-type SBW25 and Wrinkly Spreader cells in the high-O_2 region and at the A-L interface.

3. Investigate A-L interface–cell interactions for wild-type SBW25 and the Wrinkly Spreader during the early development of biofilms.
    I. Compare the surface tension of cultures, cell-free supernatants and resuspended wild-type SBW25 and Wrinkly Spreader cells.

4. Explore the link between fitness benefits of biofilm-formation and cell localisation characteristics of Complementary biofilm-forming strain (CBFS), Viscous mass (VM) and Wrinkly Spreader cells.
    I. Determine the competitive fitness of CBFS, VM and WS mutant biofilms in pair-wise fitness assays with different initial ratios.
    II. Compare CBFS, VM and WS mutant cell distributions through the liquid column and localisation to the high-O_2 region.

3.2 Results

3.2.1 *Pseudomonas fluorescens* SBW25 wild-type and WS mutant cells are motile and aerotaxic

Swimming is considered an important trait amongst bacteria, specifically aerotaxic motility which can position cells within the high-O_2 region close to A-L interfaces (Hölscher *et al*., 2015). It is predicted that wild-type SBW25 and WS mutants are both motile and aerotaxic, as cells migrate upwards in static liquid microcosms (Spiers and Rainey, 2005) and an aerotaxis sensor is annotated within the genome (Silby *et al*., 2009). To confirm flagella mediated motility in wild-type SBW25 and the WS mutant, replicate (n = 8) swimming assays utilising semi-soft agar were undertaken. A non-swimming flagella-deficient *fleQ*$^-$ mutant and chemotactic mutant *che*A$^-$ were used as controls, where no swimming migration from the inoculation point was expected for the *fleQ*$^-$ mutant, and random directional swimming for the *che*A$^-$ mutant. The mean swimming diameter was found to be significantly greater between wild-type SBW25 and WS mutant cells and the two controls, and between wild-type SBW25 and the WS (Table 3.2, TK-HSD, $\alpha = 0.05$). In addition to this quantitative comparison, a qualitative assessment confirmed chemotaxis in wild-type SBW25 and WS mutant plates, where both strains producing swimming haloes characteristic of chemotaxic cells (Figure 3.2). In comparison, the *che*A$^-$ mutant non-directional swimming through the plate, characteristic of non-chemotactic motility, and *fleQ*$^-$ cells were non-motile and did move out from the inoculation point.

Table 3.2 Mean swimming diameter (mm). This data has been used in our Jerdan *et al.* (2019) publication.

Strain	Diameter (mean mm)	Standard deviation
Wild-type SBW25	19.7	2.0
Wrinkly Spreader	11.4	1.2
***cheA*$^-$**	4.4	0.5
***fleQ*$^-$**	2.6	0.5

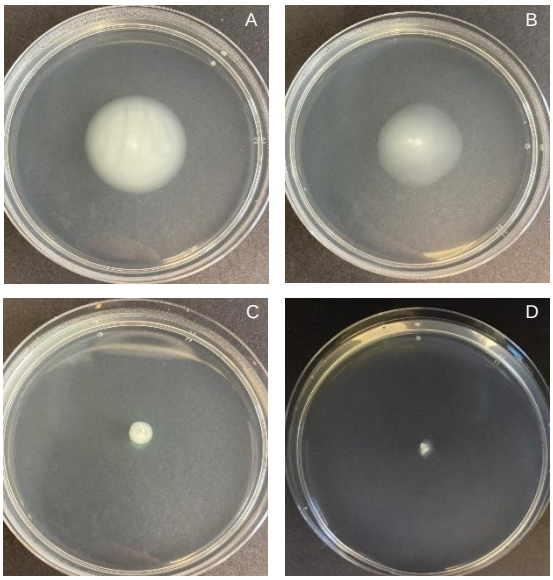


Figure 3.2. *Pseudomonas fluorescens* SBW25 wild-type and WS mutants are motile and demonstrate chemotactic motility. Swimming plates containing 0.3 % w/v agar were used to confirm that the flagella-deficient mutant *fleQ*$^{-}$ mutant was non-motile (D), *che*A^{-} mutant was motile but not chemotaxic (C) and wild-type SBW25 and WS mutants are motile (A and B), with swimming haloes characteristic of chemotactic motility.

Many pseudomonads are expected to be aerotaxic, however little experimental verification of this is available even for model strains. A qualitative system was developed to observe if *P. fluorescens SBW25* cells moved towards O_2. In replicate test tubes (n = 4) cell pellets with the metabolic indicating dye Tetrazolium Violet (TV) were set between two 5ml columns of semi-soft agar. Once an O_2 gradient is established through metabolic activity, cells had the option to move both down the soft agar column to the bottom of the test tube, or up towards the high-O_2 A-L interface. Movement upwards (but not downwards) would suggest aerotactic motility. After 48 hrs cells had migrated upwards into the top 5 ml soft agar column, and onto the surface of the agar (Figure 3.3 C and D), indicative of aerotaxic motility.

To confirm these qualitative observations, cell movement towards the A-L interface was quantified by developing a microscope slide-based assay. Microscope slides were prepared by sealing three of the four edges of the cover slip to create one side that remained exposed to air. When placed in N_2, no O_2 gradient will be established therefore cells utilising aerotaxis should move randomly throughout the slide. When left to equilibriate in air, an O_2 gradient will be re-established and aerotaxic cells are expected to move along the O_2 gradient and

accumulate at the side which is exposed to air. The *fleQ*$^-$ mutant is non-motile so are unable to move along an O_2 gradient to access O_2 even if O_2 sensing was functional. The *fleQ*$^-$ mutant cells are expected to randomly diffuse throughout the slide, with no significant increase in the number of cells found at the A-L interface. The number of cells at the A-L interface were counted after exposure to N_2 and after slides were left to equilibriate with O_2.This gives an O_2/N_2 ratio, and a value of one or below would suggest non-aerotaxic motility, and significantly higher than one suggest cells actively moving toward the A-L interface when O_2 is present. A significantly greater number of wild-type SBW25 cells were found near the A-L interface in the presence of O_2 compared to N_2, with a O_2 / N_2 cell ratio number of 1.6 ± 0.2, significantly greater than 1 (T-test $P < 0.05$) (Figure 3.3, A and B). Similar to wild-type SBW25 cells, WS mutant cells had an O_2 / N_2 cell ratio of 1.4 ± 0.1 (T-test $P < 0.05$), suggesting both are aerotaxic. Similar to plate-based motility assays, there are differences in aerotaxis motility between WS and wild-type SBW25 with wild-type SBW25 demonstrating stronger aeroatxis. In contrast, *fleQ*$^-$ mutant cells produced a ratio close to one (1.1 ± 0.1, T-test $P = 0.33$), confirming that they were not capable of aerotaxic motility and diffused randomly throughout the microscope slide.

Finally, the O_2 gradient with static cultures was visualised using a redox dye. Methylene blue is decolourised under anaerobic conditions, and in static cultures of wild-type SBW25 and WS mutants only the top of the liquid column remained blue (Figure 3.3, C).This confirmed earlier work which describes the O_2 gradient generated in static cultures of *P. fluorescens* SBW25 (Koza *et al*., 2001). By confirming aerotaxis, wild-type SBW25 and WS mutant cells should be capable of localisation within the high-O_2 region with static liquid microcosms.

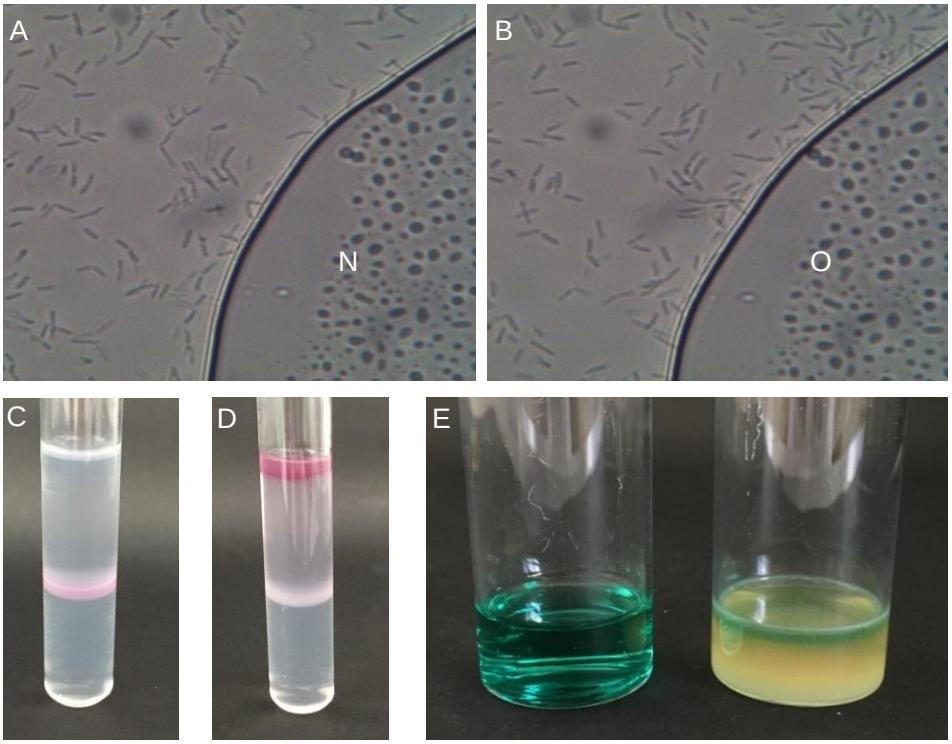


Figure 3.3. Wild-type SBW25 and WS mutant cells are aerotaxic and move along an O_2 gradient, toward the high-O_2 region. Cells are positioned randomly throughout the liquid culture on a microscope slide in the presence of N_2 as no attractant is present (A). When O_2 is re-introduced an O_2 gradient is created and a higher proportion of cells are position along the A-L interface, towards the source of O_2 (B). The movement upwards towards O_2 was observed in semi-soft agar test tubes, where cells migrated upwards from the cell pellet set within the middle of the agar column (C and D). The O_2 gradient within the microcosm system can be visualised using methylene blue redox indicator, showing the high-O_2 region directly below the A-L interface. These images have been used in the Jerdan *et al*. (2019) publication.

3.2.2 The WS mutant biofilm-forming strategy is more successful in recruiting cells to the high-O_2 region compared to flagella-mediated aerotaxic motility.

The O_2 gradient created in SBW25 cultures results in the top ~200 µm of the liquid column remaining rich in O_2 (Koza *et al*., 2017), producing a high-O_2 region directly below the A-L interface. The liquid column below this region is depleted in O_2 creating a low-O_2 region making up ~90% of the liquid column (Koza *et al*., 2011). Cells must migrate upwards to

access O_2, enriching this region with cells. As cell enrichment within this region increases, a higher proportion of the population can access higher levels of O_2 resulting in a higher growth rate. WS mutants outcompete wild-type SBW25 in static liquid microcosms (Rainey and Travisano, 1998), and it is hypothesised that WS mutants gain a growth advantage due to better access to O_2. It is expected that the biofilm-forming strategy utilised by the WS mutant is more successful in localising cells to the A-L interface.

A cell localisation assay was developed to quantify cell distribution (OD_{600}) throughout the liquid. The top 1ml represents the high-O_2 region and includes any biofilm material, and in total six samples were taken sequentially down the liquid column. Replicate populations (n = 5) of wild-type SBW25, WS mutant and *fleQ*$^-$ cells were inoculated at the bottom of the liquid column, incubated statically for 24 h, and sequential OD_{600} measurements were taken down through the liquid column. Results are shown as the relative cell density (Figure 3.4). Wild-type SBW25 and WS mutant populations show significant cell enrichment at the top 1ml compared to the rest of the liquid column (wild-type SBW25 top 1 ml 1.66 ± 0.09 and bottom 1 ml 1.04 ± 0.09; WS mutant top 1 ml 3.43 ± 0.09 and bottom 1 ml 0.7 ± 0.06, relative OD_{600}, TK-HSD, α = 0.05). The non-motile *fleQ*$^-$ strain demonstrated no significant cell enrichment at the top of the liquid column, with a significant proportion of cells remaining at the bottom of the liquid column (*fleQ*$^-$ top 1 ml 0.83 ± 0.03 and bottom 1 ml 1.79 ± 0.02 relative OD_{600}, TK-HSD, α = 0.05). This confirms that swimming motility is required to locate cells within the liquid column for cell enrichment at the top to be achieved. Significant differences in cell distributions were also seen between strains at both the top and middle of the liquid column (Figure 3.5). WS mutant static cultures had a significantly higher cell enrichment at the top of the liquid column compared to wild-type SBW25 and *fleQ*$^-$ cultures (WS 1.97 ± 0.02, wild-type SBW25 1.60 ± 0.1 and *fleQ*$^-$ 0.16 ± 0.01, relative OD_{600}, TK-HSD,α = 0.05). Wild-type SBW25 and *fleQ*$^-$ show a significantly higher cell enrichment within the liquid column (calculated as the mean of relative OD600 for the 2nd – 5th samples) compared to WS mutant cultures (WS 0.46 ± 0.01, wild-type SBW25 0.82 ± 0.02 and *fleQ*$^-$ 0.81 ± 0.01, relative OD_{600}, TK-HSD,α = 0.05).

These results confirm WS mutant cells are capable of greater cell localisation to the high-O_2 region than wild-type SBW25 cells. Both strains are capable of swimming motility and aerotaxis, but not to the same level and cell distribution throughout the liquid column is significantly different. WS mutant cells can achieve higher cell localisation to the high-O_2 region, suggesting more cells can interact and access the A-L interface.

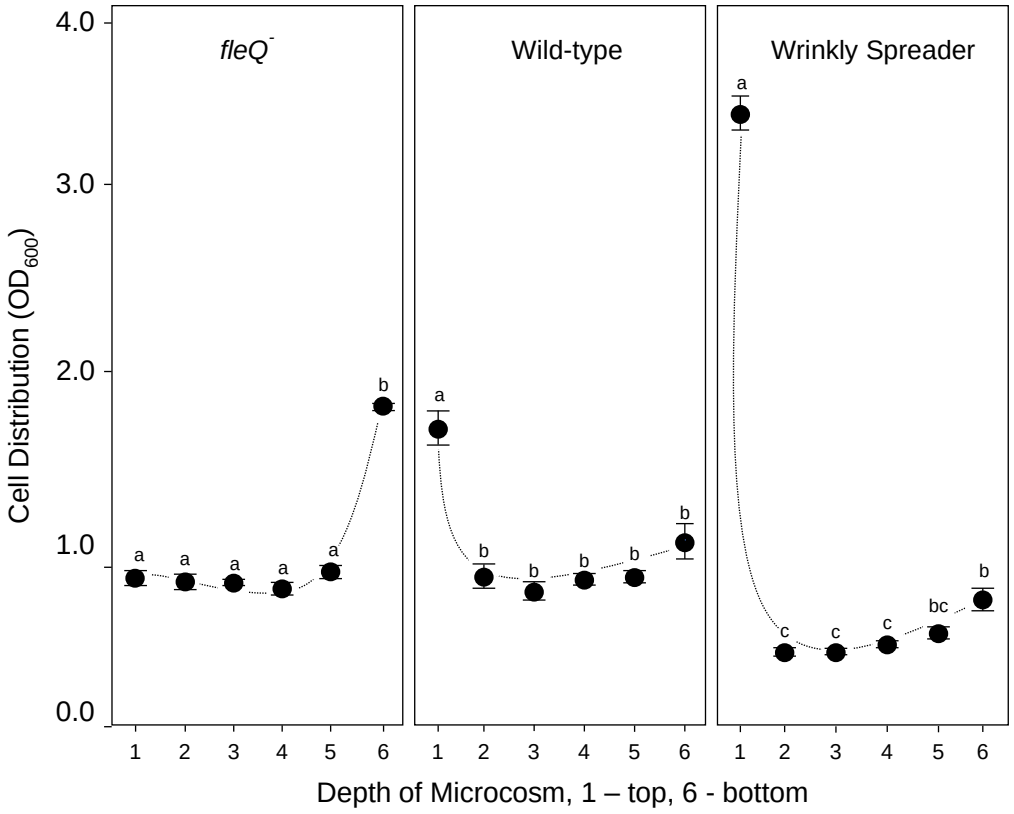


Figure 3.4. WS mutant and wild-type SBW25 cells enrich at the top of the liquid column in the high-O_2 region while *fleQ*$^-$ cells remain lower down. A comparison of cell distributions (relative OD_{600}) throughout liquid column shows WS mutant and wild-type SBW25 cells are capable of significant cell enrichment of the top of the liquid column in static liquid cultures, compared to the rest of the liquid column. The flagella deficient *fleQ*$^-$ mutant was not capable of enrichment confirming swimming motility is needed to access the high-O_2 region. Means ± SE are shown (n = 5) and means not linked by the same letter are significantly different (TK-HSD, α = 0.05). Trend lines (dashed curves) are descriptive only. This data has been used in the Jerdan *et al*. (2019) publication.

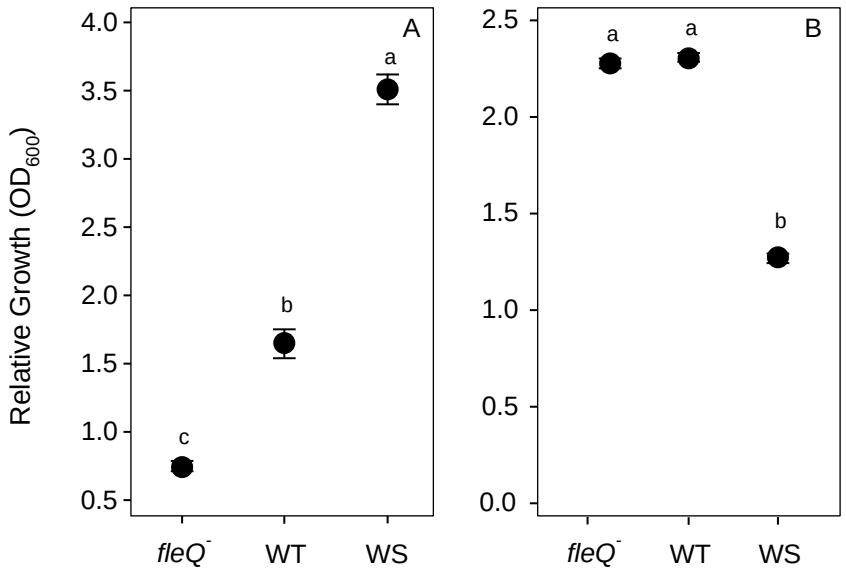


Figure 3.5. WS mutant cells show greater enrichment at the top of the liquid column in the high-O_2 region than wild-type SBW25 and *fleQ*$^-$ mutant cells. Relative distributions (relative OD_{600}) in the top 1 ml of the liquid column (A) and the middle column (mean of the 2nd ml – 5th ml (B)) was compared between wild-type SBW25, WS mutant and *fleQ*$^-$ static cultures after 24hr static incubation. WS mutants show a significantly higher cell localisation of the top 1ml. Means ± SE are shown (n = 5), means not linked by the same letter are significantly different (TK-HSD α = 0.05).

3.2.3 Overcoming physical displacement is key to maintaining cells within the high-O_2 region.

Brownian motion, vibrations and thermal or bioconvection currents (physical displacement) can act as physical forces within bodies of water effecting the position of bacterial cells. Within the *P. fluorescens* SBW25 system physical disturbance by vibrations, gentle shaking or mixing is known to cause biofilms to collapse, especially the poorly attached wild-type SBW25 VM biofilm (Koza *et al*.,2009). However, the effect of other forces collectively known as physical displacement is unknown within this system. Brownian motion displaces cells and randomises the direction of swimming (Berg, 1993) and bioconvection currents can displace cell away from the A-L interface downwards through the liquid column (Hill and Pedley, 2005). Within the *P. fluorescens* SBW25 static liquid microcosms these forces are likely to effect cell localisation at A-L interface region where O_2 is high, displacing cells further into the liquid column where O_2 is limiting. To address the presence of bioconvection within this system, qualitative assessments were made for the visual presences of bioconvection 'plumes' (long cloud of microorganisms spreading downward from the A-L interface) and the density of wild-type SBW25 were calculated. For bioconvection to occur

the density of microbial cells need to be greater than that of the liquid medium they occupy (Wager, 1911; Pedley and Kessler, 1992).

Finally, cell localisation was tested in microcosms with increasing viscosity. As agar and polyethylene glycol (PEG) increase the intermolecular friction and networks (Armisen and Galatas, 2009; Zhao, Dimova and Liu, 2015), is predicted that this will decrease the effects of physical displacement. Under standard KB* static microcosm conditions WS mutants are known to have a considerable fitness advantage over wild-type SBW25, however recent research (A. Kuśmierska, M. Petric; Speirs' research group) found this fitness advantage decreased in microcosms modified by increased viscosity. To determine the link between viscosity and fitness, it was hypothesised that the loss in WS mutant fitness is caused by an increased cell localisation to the A-L interface by wild-type SBW25. It is expected that bioconvection and Brownian motion cause physical cell displacement, within this system, by displacing cells away from the A-L interface in wild-type SBW25 static cultures. This would offer an explanation to the underlying need for biofilm-formation, in overcoming physical displacement, and increasing cell localisation at the A-L interface.

For bioconvection to occur the density of microbial cells need to be greater than that of the liquid medium they occupy (Wager, 1911; Pedley and Kessler, 1992). The density of wild-type SBW25 cultures were experimentally determined in replicate (n = 5) at a mean of 1.3 ± 0.1 g/cm^3. This confirms wild-type SBW25 cells are denser than KB* media, determined at 1 ± 0.0 g/cm^3 (K-W, P = 0.01), so bioconvection is possible. Qualitative assessment of wild-type SBW25 and WS mutant cultures (standard KB* microcosms and larger 40 ml petri dishes) took place over 6 hrs, examining the presence of bioconvection in the form of finger like plumes. No obvious plumes were observed by eye within this system, however more specialised camera or video microscopy may be needed to visualise bioconvection. Cell distribution of wild-type SBW25 and WS mutants were compared in normal KB*-DP/T and KB*-DP/T microcosms modified by viscosity agents. Varying concentrations of agar and PEG (giving a viscosity range between 1 - 7 mPa s^{-1}) were chosen due to their ability to form intermolecular networks (agar) and increase intermolecular friction (PEG), however the concentrations still left the liquid column in a fluid state.

Cells were inoculated at the bottom of the liquid column and a modified cell localisation method was used. Cell enrichment is shown as a mixed/ bottom OD_{600} ratio (Figure 3.6), where a ratio value below one suggests fewer cells have localised up the liquid column from the initial inoculation point at the bottom of the microcosm. The addition of agar and PEG at

medium and high concentrations significantly changed wild-type SBW25 and WS mutant cell distribution compared to standard KB*-DP/T microcosms after 24 hrs of static incubation. A 2x increase in the number of cells situated in the liquid column rather than the lower liquid column for both wild-type SBW25 and WS mutants in the high viscosity environment (wild-type SBW25 KB*DP/T 1.2 ± 0.2 and high PEG 2.4 ± 0.4; WS mutant KB*DP/T 4.03 ± 0.7 and high agar 8.9 ± 1.7, cell enrichment OD_{600}, TK-HSD, α = 0.05). The effect of viscosity plateaus at 3 mPa s^{-1} after which no further effect is seen in wild-type SBW25, and results are similar regardless of whether agar or PEG is utilised to increase viscosity. These results suggest when physical cell displacement is lessened with the presence of viscosity agents, both wild-type SBW25 and WS mutant populations can achieve higher cell localisation to the A-L interface. This allows wild-type SBW25 to localise more cells to the A-L interface, so can better compete for faster growth at the high-O_2 region.

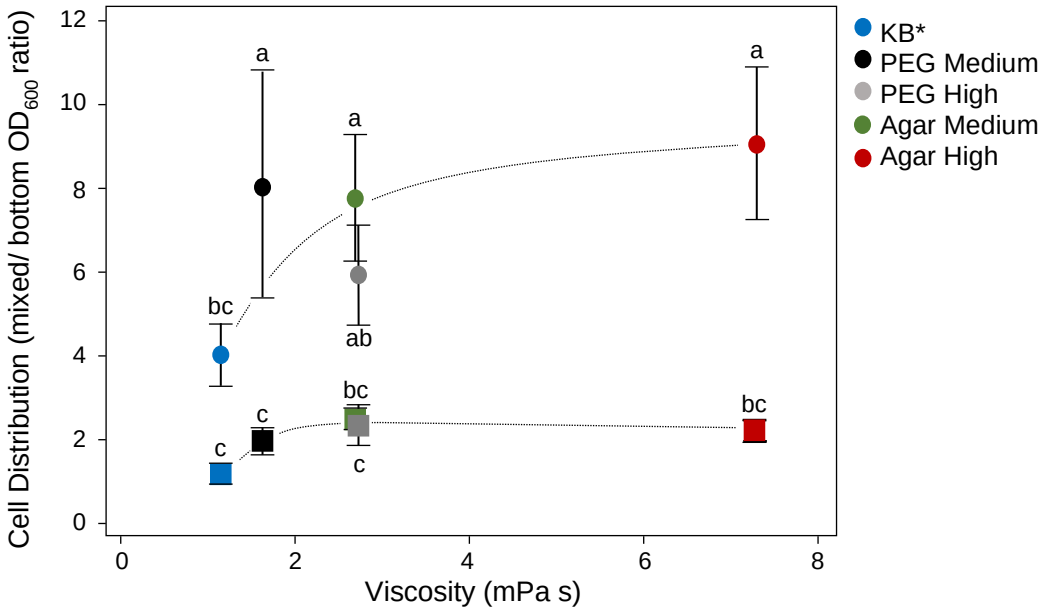


Figure 3.6. Wild-type SBW25 and WS mutant cell localisation to the high-O_2 region is increased with the addition of viscosity agents. Standard KB*-DP/T microcosm (blue) were modified with PEG at medium 2.5% (black) and high 5% (grey) concentrations and agar at medium 0.05% (green) and high 0.1% (red) concentrations. Cells were placed at the bottom of the liquid column and cell enrichment (OD_{600}) is shown here as OD_{600} of the bottom of the microcosms divided by the OD_{600} of the remaining culture after vigorous mixing (Mixed/ Bottom OD_{600} ratio). An increase in ratio suggests more cells have migrated to the upper liquid regions compared to the initial inoculation site at the bottom of the vial. As viscosity increased both WS mutants (circles) and wild-type SBW25 (squares) cell localisation increased, plateauing at a viscosity of approximately 3 mPa s. Means ± SE are shown (n = 5) and means not linked by the same letter are significantly different (TK-HSD, α = 0.5). Trend

lines (dashed curves) are descriptive only. This data has been used in the Jerdan *et al*. (2019) publication.

3.2.4 Wrinkly Spreader mutant cells are better able to access the A-L interface for biofilm-formation

Biofilms produced by the WS mutant are dry in nature, appearing to form above the A-L interface. An additional mechanism between the WS mutant biofilm, and the VM biofilm produced by wild-type SBW25 is needed to explain the appearance and location of both biofilms, in addition to an increase cellulose and attachment factor in WS mutant cells. Interfacial tension at the interface between air and liquid, better known as surface tension (ST), is a thick molecular layer that is difficult to break, however many organic molecules are able to lower tension in order to break or penetrate. It is expected that WS mutant cells poses and additional surface lowering mechanisms allowing cells to penetrate through and be positioned at the dry-side of the interface. To investigate if this could be a further key adaptive change in WS mutant cells the surface tension of overnight cultures, supernatant and washed cells were compared for WS mutants and wild-type SBW25.

Replicate overnight cultures (18 h, n = 4) were measured using a tensiometer to compare the ability to lowering surface tension (γ, mN m^{-1}). Initial ANOVA did not show any significant differences, however, this may be due to the relatively large variation within the data set and small sample size. The sample size could not be increased due to the sensitivity of the tensiometer creating a large batch effect between sampling days, so all samples being compared had to be analysed on the same day in which the maximum number of samples were tested. Results were therefore compared using a T-test and a significant difference in the ST between wild-type SBW25 cultures (32.9 ± 0.4 mN m^{-1}) and WS mutant cultures (31 ± 0.4 mN m^{-1}) were observed (P = 0.02), however not between the cell free supernatant (P = 0.18) or washed cells (P = 0.16) (Figure 3.7). To confirm this result, non-parametric analysis was completed and was coherent with initial analysis (K-W; cultures, P = 0.03; supernatants, P = 0.14; cells, P = 0.29). This result suggests WS mutant cells have an additional ability, in the form of a surface-acting-agent, loosely attached to cells, to further lower ST and penetrate across the A-L interface, allowing for subsequent biofilm development.

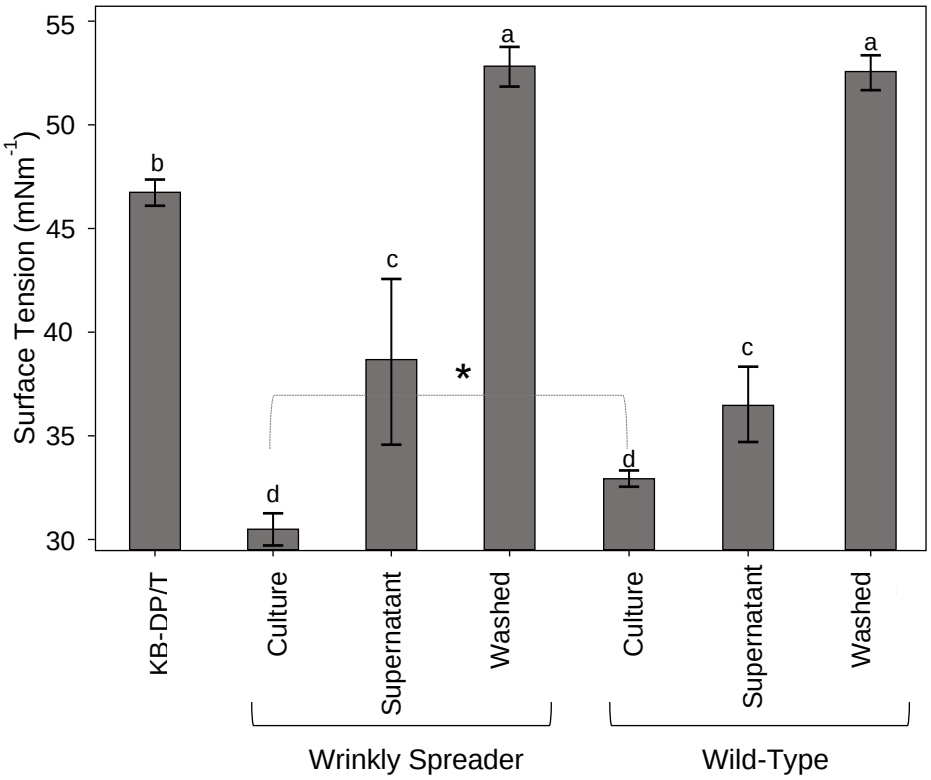


Figure 3.7. Differences in surface tension between the WS mutant and wild-type SBW25 cultures, supernatants and washed cells. Surface tension (γ, mM m-1) measurements were compared between WS mutants and wild-type SBW25 to investigate the ability to associate or penetrate the A-L interface caused by a cell surface associated feature. Means ± SE are shown (n = 4), results not connected by the same later are significant different (TK-HSD, α 0.05). Further statistical analysis show a significantly difference (*) between wild-type SBW25 and WS mutant cultures (T-test, *P* = 0.02), however not between vigorously washed cells (T-test, *P* = 0.18) or supernatants (T-test, *P* = 0.16). This data has been used in the Jerdan *et al*. (2019) publication.

3.2.5 Differences in cell localisation in biofilm-forming strains result in changes in competitive fitness

Multiple mutant strains within the *P. fluorescens* SBW25 lineage can from A-L interface biofilms, which differ in biofilm characteristics (Table 3.1). WS and CBFS mutants form strong physically cohesive class biofilms, and wild-type SBW25 cells produces a weak viscous mass (VM) biofilm that is poorly attached. All three strains possess a fitness advantage over the non-biofilm-forming wild-type SBW25, however fitness differences between these strains are unknown. The cell localisation assay developed to compare WS mutants and wild-type SBW25 cell distribution throughout the liquid column concluded that

biofilm-formation was the optimum strategy for localising the highest proportion of cells to the A-L interface. Both CBFS mutants and the VM biofilm also produce turbid liquid columns in addition to A-L interface biofilms. This suggests these mutants inhabit both regions, unlike the WS mutants, where a proportion of the population remain in the low-O_2 region. Cell localisation and competitive fitness of the WS, VM and CBFS strains were compared, to determine if fitness differences existed, and if these differences could be explained by cell localisation. It was expected that WS mutants would retain a fitness advantage over the other mutants due to increased cell localisation to the high-O_2 region.

As all three strains have distinct colony morphologies pair-wise competitive fitness assays were carried out, with variations in the starting cell ratios. Starting ratios consisted of 1:1 (or as near to 1:1 as possible) to represent two strains appearing in a population at the same time, and ratios of 1:1000 and 1000:1, where the strain inoculated at the smaller volume represents a new mutant appearing in an already established population. Fitness (W) was calculated using the ratio of Malthusian parameters (Lenski *et al*., 1991). At an inoculation ratio of near one CBFS had a significant fitness advantage over the WS mutant (CBFS W = 1.24 ± 0.05 and WS W = 0.80 ± 0.05) and the VM biofilm (CBFS W = 1.07 ± 0.03 and VM W = 0.93 ± 0.03) and the VM biofilm had a significant fitness advantage over the WS mutant biofilm (VM W = 1.14 ± 0.06 and WS W = 0.88 ± 0.06, TK-HSD, $\alpha = 0.05$). All strain combinations demonstrated negative-frequency-dependent fitness, with the rare strain gaining a significant fitness advantage as it was only competing with one other strain, while the dominant strain had within species competition for O_2 and nutrients (Figure 3.8).

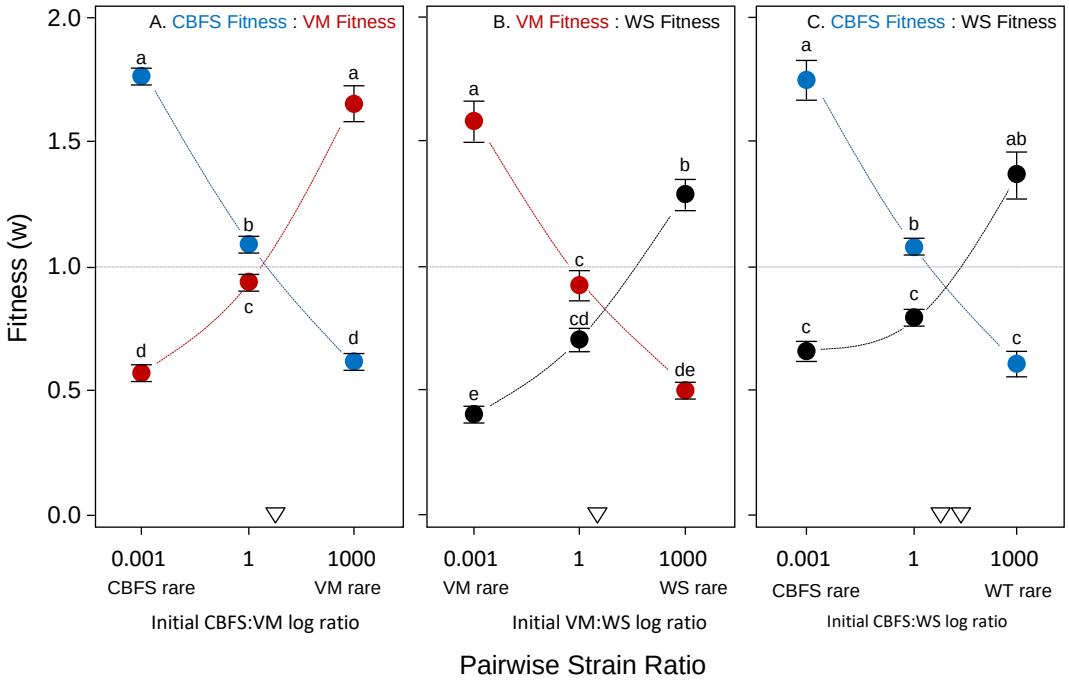


Figure 3.8. Pairwise competitive fitness between CBFS, WS mutant and VM biofilms. Pairwise fitness assays were carried out with initial cell ratios of 1:1000, 1:1 1000:1 (or close to) and cultures were incubated statically for 24 and 72 hrs before serial dilution and plating. The black dotted line at 1 indicates a fitness of 1, where the fitness of each strain would be equal. A fitness of W<1 indicates that the strain is at a disadvantage and is being out-competed by the second strain. A fitness of W> 1 indicates the strain has the competitive advantage. Means ± SE are shown (n = 5), means not connected by the same letter within the same panel and statistically different (T-K HSD, α = 0.05). Trend lines (dashed curves) are descriptive only. This data has been used in Koza *et al*. (2020) publication.

The three biofilm types were compared using the cell localisation assay in standard KB* microcosms after 24 h and 72 h static incubation, and results are shown (Figure 3.9) as relative cell distribution (OD_{600} at depth divided by total mean growth). All strains show significantly higher cell recruitment within the top 1ml compared to the liquid column, and CBFS (3.39 ± 0.01 relative OD_{600}) and WS mutant (3.55 ± 0.06 relative OD_{600}) had significantly more cell recruitment to the top 1 ml compared to the VM biofilm (1.89 ± 0.03 relative OD_{600}) after 24 hours (T-K HSD, $\alpha = 0.05$). Cell localisation to the A-L interface decreased relative to the total mean growth after 72 h in both CBFS and VM microcosms, while WS mutant cell localisation to this region increased significantly (1.2 x). The VM biofilm showed the greatest proportion of cells in the middle of the liquid column (0.78 ± 0.02 relative OD_{600}, 24 h) compared to the other strains, and CBFS had a higher proportion of

cells in the liquid column compared to the WS mutant (CBFS 0.6 ± 0.02, WS 0.39 ± 0.02 rel OD_{600}, 24 hr, T-K HSD, $\alpha = 0.05$, Figure 3.10), which was also visually observed as the CBFS liquid column was more turbid than the WS mutant. These results suggest that the optimum colonisation strategy is the ability to colonise both the A-L interface by biofilm-formation and the liquid column with free swimming cells, maximising total growth throughout the microcosm.

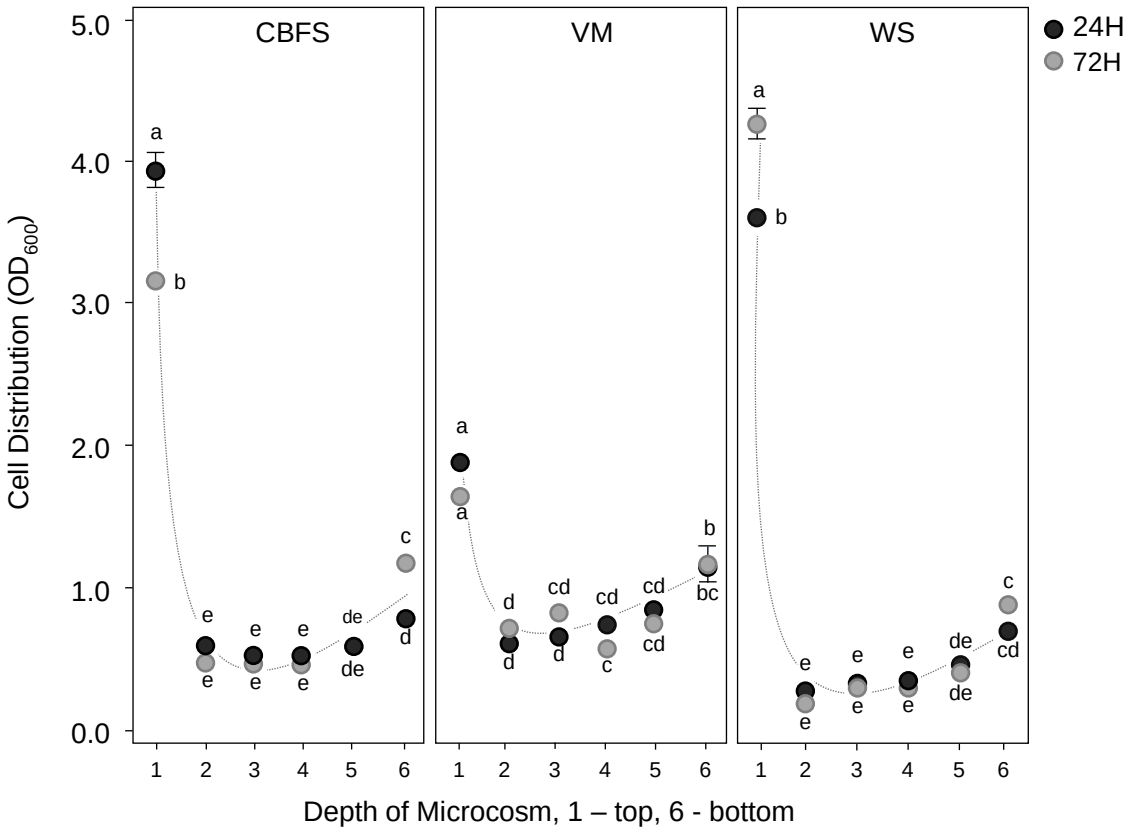


Figure 3.9. Cell distribution throughout the liquid column differs between CBFS, WS mutant and VM biofilms. Cell distribution (relative OD_{600}) was compared throughout the liquid column for CBFS, WS mutant and the VM biofilm after 24 h (black) and 72 h (grey) of static growth. Results are shown as relative growth (OD_{600} at depth divided by total mean growth). Means ± SE of the relative OD_{600} are shown (n = 5), letters not connected by the same letter and the same panel are significantly different (TK-HSD, α = 0.05). Trend lines (dashed curves) are descriptive only. This data has been used in Koza *et al*. (2020) publication.

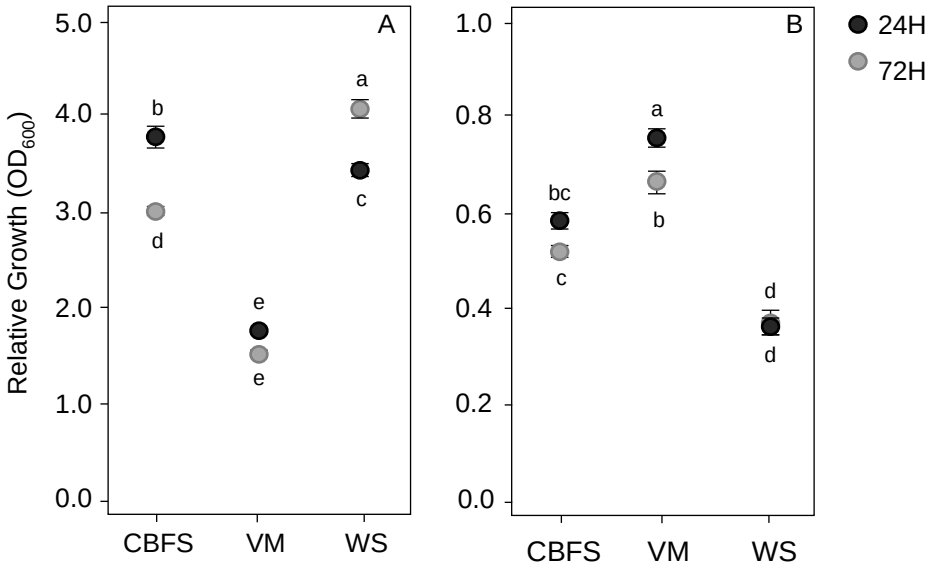


Figure 3.10. Relative growth at the top and middle of the liquid column of CBFS, WS mutant and VM biofilms. Comparison of relative growth (OD_{600}) of the top 1ml of the liquid column (A) and the middle of the liquid column (mean of the 2nd ml – 5th ml (B)) after 24 h (black) and 72 h (grey) of static growth were compared. Means ± SE of the relative OD_{600} are shown (n = 5), letters not connected by the same letter and the same panel are significantly different (TK-HSD, α 0.05). This data has been used in Koza *et al*. (2020) publication.

3.3 Discussion

The model bacterium *Pseudomonas fluorescens* SBW25 has been utilised in experimental evolution studies to explore adaptive radiation and biofilm-formation at the A-L interface. While the molecular biology behind the divergence from the non-biofilm forming ancestor into biofilm-forming mutants known as Wrinkly Spreaders is well understood, some underlying mechanisms remain un-explored. I identified three key gaps in our knowledge of this model system which have formed the basis of this research chapter and has required the development of new assays. The aim of this chapter was first to provide experimental evidence of aerotaxis in wild-type SBW25 which was believed to be a necessary behaviour that allowed cells to enrich at the top of static liquid microcosms immediately before interacting with the A-L interface to begin biofilm-formation. Secondly, the relative importance of physical displacement in the localisation of cells at the top of the liquid column and A-L interface was investigated, as the success of biofilm-formation as a colonisation strategy for the A-L interface suggests that physical displacement cannot be overcome simply by aerotaxic-directed swimming motility. Third, differences in how wild-type SBW25 and WS mutant cells access and penetrate the A-L interface were investigated, demonstrating that while both cell types can enrich at the top of static liquid microcosms, WS mutant cells still have an advantage in penetrating the A-L interface to produce biofilms. Finally, the cell-distribution assays developed here has also been applied in a comparison of CBFS, VM and WS biofilms in an attempt to understand the fitness differences between these three different structures produced by *P. fluorescens* SBW25.

3.3.1 Motility and aetrotaxis are required to enrich the high-O_2 region at the top of static liquid microcosms.

Within static liquid microcosm systems initial colonists act as ecosystem engineers by depleting O_2 in lower regions creating a high-O_2 ecological niche at the top of the liquid column (Koza *et al*., 2011). Aerobic cells within this niche benefit from an increased growth rate (Kuśmierska and Spiers, 2016) and O_2 becomes a limiting factor in the lower liquid regions of this system. Cells are required to be able localise at the top of the liquid column to be able to benefit from access to O_2, and WS mutants achieve this through biofilm-formation. However, aerotaxis motility has been evidenced to position cells at the A-L interface in other species (Hölscher *et al*., 2015), questioning the need for costly biofilm-formation. If cells utilising aerotaxis motility were capable of overcoming physical displacement and significantly enriching the high-O_2 region, then costly biofilm-formation would not be needed.

An aerotaxis assay was developed to provide experimental evidence of aerotaxis in SBW25 cells. Both wild-type SBW25 and WS mutant cells are aerotaxic, demonstrated by an increase in cell numbers adjacent to the A-L interface when O_2 rather than N_2 was present in a microscope slide based assay (Figure 3.3). These results suggest wild-type SBW25 cells can locate to the A-L interface in static liquid microcosms utilising aerotaxis motility. There were differences in both plate-based and aerotaxis motility between the wild-type SBW25 and WS mutant. This was also observed when comparing the motility of wild-type SBW25, WS mutant and the complementary biofilm-forming strain (CBFS), with likely retardation of motility through the overexpression of matrix components in WS and CBFS mutants (Koza, A and Spiers, A, Koza *et al.* 2020).

With aerotaxis now evidenced in *P. fluorescens* SBW25, the success of this strategy in localising and retaining cells within high-O_2 liquid region could be explored. For wild-type SBW25 cells to benefit from the fitness advantage associated with the high-O_2 conditions at the top of the liquid column cells must remain in place, by overcoming physical displacement and retaining cells within this region. WS biofilms are located at the A-L interface, however wild-type SBW25 planktonic cells remain unattached in the liquid region, so are expected to succumb to physical displacement resulting in a larger proportion of the population in the low-O_2 liquid region. A cell localisation assay was developed, measuring cell distribution sequentially down the liquid column. The flagella-deficient mutant *fleQ*$^-$ was used as a non-aerotaxis control, and a significant proportion of cells remained within the lower liquid column, with no significant localisation to the top of the liquid column below the A-L interface. This also confirms that swimming motility is needed to localise cells up the liquid column to the high-O_2 region.

Wild-type SBW25 and WS mutant demonstrate that both cell types can locate a high proportion of cells to the high-O_2 region. However, the WS mutant can recruit a significantly higher proportion of cells to this region through biofilm-formation, compared to wild-type SBW25 utilising only aerotaxis. (Figure 3.5). Wild-type SBW25 static cultures retained a higher level of cell density throughout the liquid column, suggesting physical displacement is displacing cells from the top of the liquid column, down into the lower liquid region where O_2 is limiting. This suggests that once WS mutant cells are positioned within a biofilm cell movement away from the high-O_2 region is restricted by EPS, caused by di-GMP signalling which turns off motility, and turns on EPS production (Ha and O'Toole, 2015). Wild-type SBW25 cells have lower localisation to the high-O_2 region comapred to WS mutant cells, despite demonstrating stronger aerotaxis. This suggests an additional step or mechanisms is needed for further cell enrichment to the high-O_2 region to occur. I suspect that WS mutant

cells can interact more efficiently with the A-L interface, allowing cells to access the interface for biofilm-formation resulting in higher cell localisation to the top of the liquid column.

Aerotaxis is thought to be a common trait amongst aerobic bacteria based on genetics, however surprisingly little experimental evidence of aerotaxis is available. Methods within the literature vary, with some developing a chemotaxis capillary assay, utilising capillary tubes to determine the rate of bacteria accumulation when O_2 is present (Barak *et al*., 1982; Alder, 1973). Others are much more complex and utilise specialised experimental set-ups, such as fluorescent spectrometry or computer-controlled gas mixer and microfluidic devices (Shitashiro *et al.,* 2003; Alder *et* al., 2012). Within this research a microscope slide-based approach has been used, and although limitations clearly exist as the O_2 gradient could not be quantified, these problems can also occur within more complex assay systems (Alder *et al*., 2012). However, within a more simplistic approach, this experimental set-up is more readily available, and the wider research community could apply to provide further published data of aerotaxis motility in a wider range of bacterial species.

WS mutant cell recruitment to the A-L interface has been compared to other mini-transposon (mini-Tn*5*) WS mutants using a cuvette based recruitment assay (Spiers and Rainey, 2005). The localisation assay developed here provides further evidence of the ability of WS mutant cells to localise at the top of liquid columns, but also determines the nature of cell distribution though the entire liquid column in both WS mutants and wild-type SBW25 static cultures. Cells have to utilise aerotaxis motility to migrate along the O_2 gradient from the bottom of the liquid column to the high-O_2 region below A-L interface, and this assay demonstrates the ability of each strain to localise and retain cells within this region. However, as cells were vortexed and re-suspended to form a dense cell pellet placed at the bottom of the microcosms vial, some cell debris or dead cells may remain here. This may explain the small increase in cell localisation found in the 6^{th} sample taken from the microcosms. Therefore, when comparing cell localisation in the low-O_2 region the 2^{nd} – 5^{th} samples were chosen to represent cell localisation in this region.

The cell localisation assay developed here, has provided evidence of the nature of cell distribution in both wild-type SBW25 and WS mutant static liquid microcosms. This assay demonstrates the extend of cell localisation within the low-O_2 column in wild-type SBW25 and how successful WS mutants can localise a much higher proportion of cells to the high-O_2 at the top of static liquid microcosm. This analysis also reveals further questions, as I suspect WS mutants cells possess an additional mechanism or step to achieve higher localisation by interacting and accessing the A-L interface.

3.3.2 Biofilm-formation is the optimum strategy in overcoming physical displacement and colonising the A-L interface

The physical abiotic environment can affect the movement of bacteria. Brownian motion, vibrations and thermal or bioconvection currents are collectively referred to as physical displacement and can contribute to the movement of cells throughout static liquid. Brownian motion can displace cells and randomize direction of swimming, and this can be amplified with hydrodynamic interactions between the bacterium and the boundary surface (Gaungali, Lick-Kong and JayX, 2008). Bioconvection can also displace cells away from A-L interfaces, when the accumulation of bacteria cells at the interface becomes too dense, and cells are displaced down a body of water. The aim of this section was to provide evidence of physical displacement occurring within this system, and evaluate how this displacement effects the relative success of aerotaxis and biofilm-formation in colonising the high-O_2 region directly below the A-L interface.

For bioconvection currents to occur cell density must be higher than the liquid medium they are in (Wager, 1911, Pedley and Kessler, 1992), and can often be visualised in the form of downward plumes. To determine if bioconvection currents occur within this system, microcosms and larger petri dishes incubated with wild-type SBW25 were visually monitored. Bioconvection currents, in the form of downward plume, were not visually detected within this system, however wild-type SBW25 cell density was estimated at 1.3 g/cm^3, 0.3 g/cm^3 denser than KB* media, a key component for the occurrence of bioconvection leading to the possible transportation of cells away from the high-O_2 region (Pedley and Kessler, 1992). Bioconvection currents are generally assumed to be a positive force in non-aerated environments by the transportation of O_2 down to the low-O_2 regions, however research has provided evidence to disagree with this assumption (Janosi *et al*., 2002) and with the SBW25 system the O_2 gradients persists within 5-day experiments (Koza *et al.,* 2011). The effect of bioconvection currents within this microcosms system still remains unclear. Recently, long-range bioconvection currents were shown to be caused by cellulose produced by ancestral SBW25, by the visualisation of ‘finger-like plumes’ falling from the A-L interface after 25 h of incubation using a specialist camera (Andre, Dufour and Rainey, 2019). It is important to note this was recorded in 20ml KB column, a much larger environment than the 6ml liquid column of static liquid microcosms. With the specific conditions needed for the occurrence of bioconvection, it is inconclusive if they are present within this 6ml system. The specialised equipment for viewing bioconvection would need to be applied to this specific system before concluding that bioconvection currents occur. Other

factors contribute to the occurrence of bioconvection, including evaporation and salt accumulation at A-L interface (Dunstan *et al*., 2018), suggesting bioconvection may be a more common occurrence. However, Brownian motion is a common force in moving bacterial cells and has been evidence to randomise and rotate the running paths in other bacterial species (Mitchel and Kogure, 2005).

Along with other displacement caused by knocks or vibrations, physical displacement is likely to occur within static liquid microcosms. The impact of these forces were then explored further by eliminating or lessening physical displacement, and exploring the difference in cell localisation to the high-O_2 region in an altered environment.

Wild-type SBW25 cells remain unattached within the liquid, so are likely to succumb to physical displacement, with cells being pushed away from the A-L interface and into the lower liquid region. This would result in a high proportion of cells in the middle of the liquid column, which was demonstrated by the cell localisation assay. To further investigate the effect of physical displacement, the cell localisation assay was modified for microcosms with added viscosity. Agar and PEG act as viscosity agents by increasing intermolecular friction and networks (Armisen and Galatas, 2009, Zhao, Dimova and Liu, 2015). Preliminary research using microcosms modified with viscosity agents found the WS mutant competitive fitness decreased when viscosity increased (A. Kuśmierska, M. Petric; Spiers' research group). I predict that the introduction of viscosity limits physical displacement within the system, lessening the effects of physical displacement. This would allow wild-type SBW25 cells to achieve higher cell localisation with the high-O_2 without displacement away from this region. Wild-type SBW25 cells should therefore be better able to compete with the WS mutants as a result of better localisation to the high-O_2 region resulting in increased growth rates. The preliminary work undertaken within the Spiers' research group confirmed the viscosity (mPa s) for low, medium and high concentrations of agar and PEG using rheometry, and the growth or inhibitory effects of PEG on *P. fluorescens* SBW25. Some bacterial species including pseudomonads can use PEG as a sole carbon source promoting growth (Haines and Alexander, 1975; Obradors and Aguilar, 1991; Hu *et al*., 2007). No growth promoting effect was found, however there was a slight toxic effect in relative growth when PEG was at higher concentrations.

The addition of viscosity to static liquid microcosms resulted in an increase in cell localisation of both wild-type SBW25 and WS mutant static cultures (Figure 3.6). Wild-type SBW25 cells were better able to localise and retain cells with the high-O_2 liquid region compared to

standard conditions, confirming the negative effect of physical displacement on wild-type SBW25 colonising the A-L interface through aerotaxis. WS mutant cells also show an increase in cell localisation suggesting biofilm-forming strains also benefit when physical displacement is reduced. Cells are better able to colonise the A-L interface and a greater proportion of cells reach this region, resulting in an increase population growth at the A-L interface. Cell localisation plateaued when viscosity reached ~3mPa s, and a decrease in localisation in high PEG microcosms is in agreement with initial analysis of the slight toxic effect of PEG at a high concentration. An increase in wild-type SBW25 cell localisation to the high-O_2 region provides evidence for the reduction of the WS mutant competitive fitness in viscous microcosms, as wild-type SBW25 cells are better able to compete for O_2 at the A-L interface niche. It may also be that WS mutant cells respond differently to drag or sheer forces, however as cellulose expression is constant and at higher levels in the WS mutants this is less likely.

P. fluorescens SBW25 has one of the fastest swimming velocities of the *Pseudomonas* genus, and evidence of aerotaxis suggests cells can overcome physical displacement. This was recently confirmed by taking the diffusion (effective) coefficient for motile bacteria (D_B) and the maximum swimming speed and average time of runs, calculating that continuous aerotaxis swimming would be sufficient in overcoming physical displacement and retaining cells within the high-O_2 region (A. Spiers; Spiers' research group). However, to continually overcome physical displacement cells would require constant motility to remain within the high-O_2 region. WS mutant cells located within a biofilm do not require further movement to retain position, however initial biofilm development is costly. For biofilm development to occur, initial WS mutant altruists require energy to produce cellulose, the main extracellular polymeric substance (EPS) of WS mutant biofilms, by the polymerisation of UDP- glucose (Spiers *et al*., 2002). A resource trade-off (Arcerenza, 2016; Ferenci, 2016) is costly to initial colonists and initial growth rates may drop, however the cost benefit outcome results in future generations benefiting from an increased growth rate within the biofilm at the high-O_2 region. *Pseudomonas aeruginosa* PA01 and *Vibreo cholerae* C6706 also demonstrate this biofilm-formation resource trade-off (Yamamoto *et al*., 2011; Nadell and Bassler, 2011). This suggests the initial cost of biofilm-formation outweighs the continual cost of cell motility to remain within the high-O_2 region (Josenhans and Suerbaum, 2002). Wild-type SBW25 cells that momentarily switch off swimming are displaced down the liquid column by physical displacement, requiring continuous swimming to retain position. Further exploration by quantifying total energy expenditure of both strategies would confirm the total cost-benefit outcome of biofilm-formation versus aerotaxis. Isothermal microcalorimeter measuring total

cell metabolism could be developed to compare wild-type SBW25 and WS mutant populations over three days of static incubation. A trial with isothermal microcalorimeter from *Symcel* was explored in 2020, but due to the COVID-19 pandemic this was put on hold.

By confirming aerotaxis and cell localisation it is concluded that biofilm-formation at the A-L interface is a more effective strategy in retaining cells within the high-O_2 region of static liquid microcosms. The underlying need and success for biofilm-formation is to overcome constant physical displacement away from the A-L interface, found in wild-type cells utilising aerotaxis to access the high-O_2 region.

3.3.3 WS mutant cells are better able to access the A-L interface

Biophysical properties of air-liquid interfaces result in tension (surface-tension, ST) created by strong hydrogen bonding between water molecules which are difficult to break. Microorganisms must possess characteristics allowing exploitation of interface properties to interact with the interface in order to attach, adhere or penetrate (Preston and Roger, 2005). Most organic solutes are capable of lowering surface tension (Kjelleberg, 1985). WS mutants can achieve much higher cell localisation to the high-O_2 at the top of static liquid microcosm compared to wild-type SBW25, and these results suggest WS mutants are better able to access the A-L interface to increase cell localisation to this region. In addition the WS mutant biofilm is visually dry compared to the VM biofilm produced by induced wild-type cells, suggest WS mutant biofilm formation occurs above the A-L interface. To test this hypothesis tensiometer analysis was carried out on wild-type SBW25 and WS mutants to determine if there are any differences in surface-active properties, allowing WS mutant cells to better interact and penetrate through the interface. WS mutant cultures were better able to lower surface tension than wild-type SBW25 cultures, confirmed by tensiometer analysis (Figure 3.7). This was not seen in culture supernatants or in washed cells. The additional lowering of 2 mN m^{-1} found in WS mutant cultures suggests a weakly associated surface-active-agent present within WS mutant cells in addition to viscosin, a surfactant lipopeptide also produced by wild-type SBW25 cells (Alsohim *et al*., 2014) which is also evidenced to lower ST (Koza *et al*. 2009, de Brujin *et al*., 2007). This would allow cells to penetrate the interface, locating cells above the interface leading to successful biofilm development. As a result cell localisation to the high-O_2 region would increase by the ability to access the A-L interface, and explains the physical dry nature of the WS mutant biofilm.

The overproduction of partially-acetylated-cellulose resulting in the WS mutant phenotype may also contribute to the WS mutant ability to lower ST and form biofilms above the interface, as cellulose is known to lower surface tension (Ardizzones *et al*., 2001; Stana-Kleinschek *et al*., 2002). Within the induced VM biofilm produced by wild-type SBW25, cellulose is responsible for the lowering of surface tension by ~3.8 mN m^{-1} (Koza *et al*., 2009), and the increased cellulose production in WS mutant cells may account for the further reduction in ST. Further experimentation would aim to identify the surface-active-agent and confirmation of its ability to lower ST, and the possible role of the cellulose concertation in lowering surface tension. This would provide a full mechanistic explanation to A-L interface colonisation in the WS mutant and highlight the ecological opportunity that exists with the ability to further lower ST at A-L interfaces.

With evidence of aerotaxis motility in *P. fluorescens* SBW25, physical displacement occurring within the system, and the ability of WS mutant cells to further lower the surface tension of the A-L interface, the original model for the diversification of *P. fluorescens* SBW25 can be developed to explain a more complex environment. Constant physical displacement drives the need for biofilm-development and the ability to penetrate the A-L interface to occupy the dry facing surface is key to successful biofilm development and increases cell localisation to the high-O_2 region (Figure 3.11).

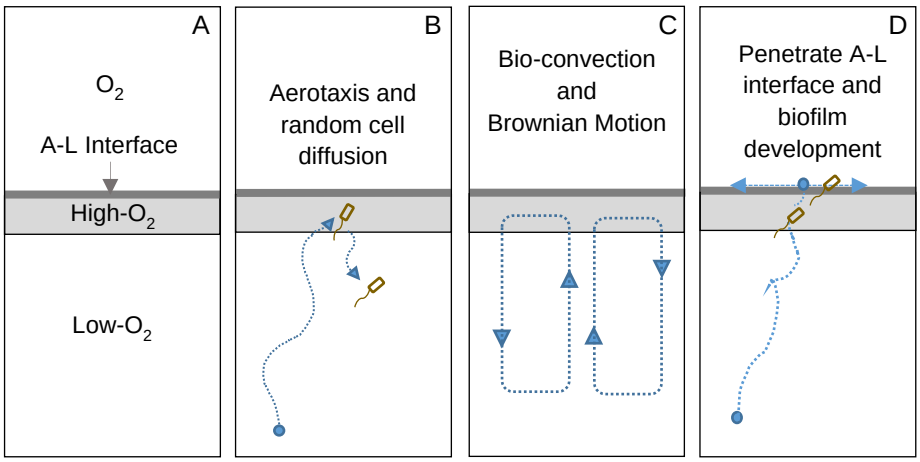


Figure 3.11. The static microcosm environment is complex and physical disturbance drives biofilm evolution. Early colonists of the static liquid microcosm act as ecosystem engineers by the up-take of O_2 in the liquid column creating an O_2 gradient and a high-O_2 ecological niche directly below the A-L interface (A). Aerotaxis and swimming motility allow wild-type SBW25 and WS mutant cells to enrich in the high-O_2 region but during moments of relaxation cells will randomly diffuse and

be physically displaced out of this region (B). Bioconvection currents and Brownian motion contribute to physical displacement, randomizing the direction of swimming motility and displacing cells down into the low-O_2 liquid region (C). Penetration of the A-L interface by lower interfacial tension allows WS mutants to initiate biofilm-development across the surface of the A-L interface, avoiding the constant need for swimming to avoid physical displacement and increase cell localisation to the high-O_2 region (D).

3.3.4 Differences in cell localisation provide fitness advantage amongst similarly capable biofilm-forming strains

The cell localisation assay developed within this chapter provides evidence that the key to the WS mutant success is increased cell localisation to the high-O_2 region through biofilm-formation at the A-L interface, with limited cell localisation in the liquid column. However, this advantage has only been compared to the non-biofilm forming wild-type SBW25. Other biofilm-forming mutants in the *P. fluorescens* SBW25 lineage also produce biofilms. The VM biofilm produced by wild-type SBW25 cells (induced by endogenous iron (Spiers *et al*., 2006; Koza *et al*., 2009; Koza *et al*., 2001) and the complementary-biofilm-forming strain (CBFS (Gehrig, 2005)) differ in biofilm characteristics and EPS. Another difference observed compared to the WS mutant biofilms, is the VM and CBFS static cultures both appear to have growth in the liquid column in addition to biofilm-development at the A-L interface (introduction table 3.1). This suggests that VM and CBFS cells are colonising both the high-O_2 region through A-L interface biofilm formation, and the lower liquid column, where O_2 and growth is lower, but makes up 90% of the microcosm environment (Koza *et al*., 2011). The three *P. fluorescens* SBW25 biofilms therefore provides a system to test if biofilm characteristics are important when competing in static liquid microcosms, or, is how a cell can utilise the entire microcosm environment important. Although cells located in the high-O_2 region receive higher growth conditions, this region is small, approximately ~200 µm, so in co-culture strains will be competing for this region. Meanwhile, the lower O_2 liquid region making up most of the microcosm environment remains underutilised but could be used to maximise productivity of the population. To determine if occupying the low-O_2 region is important, or if increased biofilm characteristics to successfully compete for the high-O_2 region, the three strains were compared using a pair-wise fitness assays and the cell localisation assay developed within this work.

The liquid column of the VM biofilm remains cloudy upon static incubation for 3 days, suggesting a large proportion of cells remain within the low-O_2 liquid niche, and a similar

observation was made with CBFS static culture, but to a lesser extent. A comparison into the cell localisation of each strain confirmed the VM biofilm had significantly higher cell localisation in the liquid column compared to the CBFS and WS mutant after both 24 and 72 h. The proportion of WS mutant cells at the A-L interface increased from 24 to 72 h, as suggested by previous experiments. CBFS cells showed a decrease in the relative proportion of cells located at the A-L interface from 24 to 72 h, and the turbid nature of the liquid column in 3 - day old cultures was confirmed by an increased cell density in this region (Figure, 3.10). As suspected, these results suggest that CBFS and wild-type SBW25 (VM biofilm) cells occupy both the high-O_2 niche, and the lower liquid region, while WS mutants have limited cells in the low-O_2 region with a significantly high proportion of the population located within the high-O_2 region.

Colony morphology between the three strains are distinct, so provides a system for pair-wise fitness comparison, to determine if biofilm characteristics or cell localisation is important when competing within static liquid microcosms. Fitness assays were compared with a 1:1 inoculation ratio, and a 1:1000, where 1 represents a rare phenotype arising in a population. Negative-frequency dependency was present when each strain was the dominant (1:1000 ratio), and the rare strain possessed a significant competitive fitness (Figure 3.8). As a strain becomes dominant it becomes 'its own worst enemy' (Lewontin *et al*., 1974) as dominance results in competition within the strain, and a rare phenotype arising within the population has an advantage as within-strain competition for limiting resources becomes dominant. Despite stronger biofilm characteristics by the WS mutant compared to the VM biofilm, which is readily destroyed by low disturbance (Koza *et al*., 2009), the VM biofilm had a significant competitive fitness advantage over the WS mutant when incubated in static microcosms at a 1:1 ratio. CBFS had the greatest fitness advantage over both strains.

These results suggest that the strong biofilms characteristics of the WS mutant does not present a competitive advantage when competing against a weaker biofilm-forming strain within the high-O_2 region and for access to the A-L interface. Rather, how a strain utilises the entire microcosm environment does. A large proportion of wild-type SBW25 and CBFS cells are located in the low-O_2 region where growth is slower, however by occupying both the A-L interface and liquid column total population productivity is maximised. The ability to colonise both niches results in a trade-off between fast growth at the high-O_2 region, and a slower but less competitive growth within the liquid column. In the event of intermediate or high levels of disturbance resulting in the destruction of the biofilm community, VM and CBFS populations can continue growth within the liquid column, characteristic of a bet-hedging approach (Jong

et al., 2011). Fitness can change when environmental and physical conditions change, demonstrated recently with WS mutant biofilm gaining a fitness advantage under low disturbance conditions, with the VM biofilm collapsing immediately (A. Koza; Spiers research group, and Koza *et al*., 2020). However, under static conditions, multiple niche-occupation is the optimum competitive strategy.

All three biofilm types enjoy a fitness advantage over the non-biofilm-forming ancestral SBW25 in pair-wise fitness assays. Further research could explore a multiple-strain fitness assays, including the non-biofilm-forming SM-13 (SBW25 *wssB*::mini-Tn*5* (Spiers *et al*., 2002)) as a reference strain. This would require further development as multiple colony morphologies on plates may become difficult to differentiate, and neutral marked strains may need to be developed. However, this would provide a more complex community model, with a comparison of varying biofilm characteristics and differences in the nature of cell localisation throughout the liquid column. This research has implications to wider biofilm research where convergent evolution can produce a variety of biofilm-forming mutants differing in fitness, and the changing environments within infections or industrial setting can favour other residing mutants with slightly different biofilm properties or colonising strategies. This research also suggests a wider view is needed when studying biofilms, as planktonic cells adjacent to a biofilm can influencing strain fitness and total productivity.

3.4 Chapter Conclusion

The aim of this chapter was to further understand the underlying need and success of A-L interface biofilm-formation by utilising the model bacterium *Pseudomonas fluorescens* SBW25 within a static liquid microcosm system. WS biofilm-forming mutants possess a fitness advantage over the archetypal non-biofilm forming wild-type SBW25 through increased O_2 access and suppression of competitors. However, aerotaxis motility is known to locate cells within the A-L interface questioning the need for costly biofilm-formation. This chapter demonstrates that *P. fluorescens* SBW25 is aerotaxic, and this cost-effect behaviour is sufficient in overcoming physical displacement and locating cells within the high-O_2 region. However, wild-type SBW25 cells that momentarily switch off motility are moved down into the low-O_2 liquid region by physical displacement such as Brownian motion and possible bioconvection currents, demonstrated by increased cell distribution throughout the lower liquid column compared to the WS mutant. This physical displacement drives the need for biofilm-formation, and the WS mutant can achieve higher cell localisation to the high-O_2 region, and the transition of WS mutant cells from planktonic state to biofilm life through

production of EPS allows cells to overcome physical displacement and remain within the high-O_2 region. Biofilm-formation is the optimum strategy in localising and retaining cells within the high-O_2 region.

The ability of WS mutant cells to achieve a significantly higher cell localisation within the high-O_2 region compared to wild-type SBW25 and the dry nature of the WS mutant biofilm suggested an additional mechanisms is needed to access the A-L interface and achieve better localisation. Tensiometer analysis suggests WS mutants possess the ability to further lower surface tension of the A-L interface, likely from a surface-active-agent loosely attached to cells capable of further lower surface tension. WS mutant cells can therefor better penetrate the A-L interface and colonise the 'air-side', explaining the dry and robust nature of the WS mutant biofilm, a key adaptive change in the WS mutant allowing for successful colonisation and retention of cells at the A-L interface compared to wild-type SBW25 populations.

Finally, whilst biofilm-formation presents a clear fitness advantage over non-biofilm formers within this system, colonisation of the liquid column is still important to maximise productivity. Although depleted O_2 conditions presents occupants with a lower growth rate, multiple-niche occupation provides a fitness advantage when competing against other biofilm-forming strains, as the A-L interface niche is highly competitive. Although WS mutants produce robust biofilms they are outcompeted by more fragile biofilm-forming strains such as the VM biofilm produced by wild-type SBW25, that also occupy the liquid column. This research highlights the importance of continuing to ask deeper questions surrounding how and why biofilms form in model bacterial population systems to further our understanding of biofilms in nature.

Work within this chapter has contributed to two publication: 'Penetrating the air-liquid interface is key to the colonization and wrinkly spreader fitness' published in *Microbiology,* August 2019 (Appendix 2) and 'Three biofilm-types produced by a model pseudomonad are differentiated by structural characteristics and fitness advantage' published in *Microbiology,* June 2020 (Appendix 2).

Chapter 4. Development of a microcosm model system to investigate selective effect of O_2 limitations on community-aggregated traits and productivity in multi-species air-liquid interface biofilm-forming communities

Abstract

Single-species studies are fundamental in our understanding of biofilm-formation and development but does not reflected the complexity of natural or managed microbial communities which may also produce biofilms. Static incubation in liquid microcosms results in a heterogeneous environment, where depletion of O_2 creates a relatively high-O_2 region directly below the air-liquid (A-L) interface, a common environmental gradient found in nature. This system has been developed for single-species studies (*P. fluorescens* SBW25 system), however in this research I develop this model system to reflect the natural complexity of multi-species communities. Using a soil-wash as an inoculum and a serial-transfer experimental design, I investigate changes in A-L interface biofilm-forming communities and productivity. Communities were incubated over 10-60 days and 49-56 'community generations' (estimated number of generations across all species across the duration of transfer experiments) with analysis of biofilm-associated traits determined at the community-level and from individual isolates from the final-transfer communities and original soil-wash inoculum. Initial analysis showed a decrease in community productivity after selection, with small increases in community biofilm strength and attachment in static transfers with increased incubation periods between transfers. Further analysis suggested that nutrient depletion and toxic waste product accumulation, and selection for more competitive phenotypes might account for the reduction in productivity. Isolate-level analysis revealed a decrease in community diversity and a biofilm-associated phenotypic shift amongst isolates recovered from the initial soil-wash and final static-transfer communities which suggested that the serial-transfer communities were subject to selection. Communities

were stratified and cell-localisation experiments showed high productivity in the low-O_2 liquid column, even within biofilm-only serial transfers. Samples taken from the biofilm and the lower liquid column were able to re-colonise both regions in fresh microcosms, indicating that community members were capable of migration which was confirmed by motility and cell localisation experiments with individual isolates. This suggests that the communities retained strains with plastic phenotypes that maximised productivity within the system, with a resource trade-off between fast growth in the high-O_2 region and slower growth in the less-competitive O_2-depleted region. This research demonstrates the complexity of multi-species biofilm-forming communities and how they respond to different selection pressures, and identifies the importance of the non-biofilm-forming 'space' on biofilm-forming communities and total productivity.

4.1 Introduction

Single-species microbial research has been fundamental in our knowledge of dynamics of microbial populations, but it fails to address the complex microbial communities that exist in nature. Single-species biofilm studies are responsible for our current knowledge of biofilm-formation, development and structure (Lenski, 2017). However, biofilms in nature are complex community assemblages of cells from multiple species living in close proximity and interacting with each other and the environment (Hall-Stoodley *et al*., 2004; Elias and Banin, 2012). Biofilm research now calls for studies to be scaled-up to provide a multi-species system, closer the natural scenario and ecologically relevant. Multi-species studies are becoming a key research model for evolutionary studies and a powerful research tool in ecology (Blasche *et al*., 2017; Kovács and Dragoš, 2019).

Ecological and evolutionary processes are known to shape community structure and community productivity in response to environmental and anthropogenic selective pressures (Kaltz *et al.,* 2012; Lawrence *et al.,* 2012; Fienga *et al.,* 2015; Castledine *et al.,* 2019). This suggests cells within biofilm communities are under constant selection pressure and must adapt to survive, changing community biofilm properties. Biofilm properties are sensitive to the abiotic or biotic surface properties colonised, nutrient availability and community composition (Stoodley *et al*., 1999). Interactions of species within influence the resulting properties and emerging functions of the biofilm (Friedman and Gore, 2016). Complex eco-evolutionary dynamics within community biofilms limit our ability to produce and control multi-species biofilms in a variety of context including the treatment of infections, bioremediation, biocontrol and plant growth, wastewater treatment and biomass conversion. It is therefore important to understand the complex successional changes which can develop our understanding of the progression of biofilm associated infections and changes in ecologically important biofilm communities in nature (Røder, Sørensen and Burmølle, 2016).

Evolutionary and ecological changes are linked and can occur within the same space and timescale (Abrams, 2001; Pelletier, Garant and Hendry, 2009). Bacteria have short generation times and populations numbers are high, so selective pressures can result in extreme effects, and these effects can be seen over a relatively short time scale. A common approach to study eco-evolutionary dynamics over various timescales is by serial transfer, developed in Lenski's *Escherichia coli* long-term evolution experiments (Lenski *et al.,* 1991). This approach captures evolutionary dynamics in real-time. Here, replicate populations of *E.coli* B were incubated for 24 hrs, after which 1 % of the population is transferred over to a

fresh environment. Each transfer represents a dispersal, pulse disturbance or re-seeding event, in which populations recover. Within microbial communities, this process is likely more complex, where community recovery results in ecological filtering and competition. During each growth period community productivity, structure and community-aggregated traits such as biofilm-formation are likely to alter through adaption and changes in community composition. Lenski's transfer experiments are still ongoing after 30 years, however eco-evolutionary dynamics and changes have been observed over a much shorter time scale. 100 – 200 day experiments have found significant divergence in microcosm populations and changes in biofilm communities (Poltak and Cooper, 2010; Johansen *et al.,* 2019), and within serial transfer experiments evolutionary and ecological changes were observed after 40 – 70 bacterial generations (Kaltz *et al.,* 2012 ; Lawrence *et al.,* 2012; Fienga *et al.,* 2015; Castledine *et al.,* 2019). Similarly, early biofilm establishment studies (1 – 7 days) show how intrinsic phenotypic traits affect succession within soil bacterium consortium (Burmølle, Hansen and Sørensen, 2007). This suggests a serial-transfer approach in static liquid microcosms will provide a selective effect in which ecological and evolutionary dynamics and changes can be captured over a short time period. The proportion of sample transfer can also influence changes, where a higher proportion of the community can be transferred to avoid a bottle neck effect.

Key to studying successional changes within microbial communities is capturing changes at both the community and individual-isolate level. Within biofilm community research this can involve investigating changes in biofilm characteristics, as well as community productivity and interaction dynamics. Focus in ecological studies is to determine taxonomic changes in community composition, by determining changes in species diversity and evenness (Wang *et al*., 2016). Molecular and metagenomic techniques can identify species composition, which can be calculated as the relevant abundance to indicate species richness and evenness (Ren *et al*., 2014). However, there is a recent shift of interest in community ecology to determine community changes through functional traits, and how individual-level traits influences the emergent community properties (Konopka, 2009). Similarly, recent evidence suggests genomics and functional diversity do not necessarily map (Kraemer *et al.,* 2010; Vos and Velicer, 2006), rather a single strain can exhibit different functional traits dependent on environmental conditions and community composition.

Important to the experimental design of multi-species community biofilm model studies is the selection an appropriate model community. Poly-microbial model communities can range from a dual-strain system of two know laboratory strains, to natural sources of microbial

communities from soil or streams, where there is an assumption of coexistence between species within the sample (Blasche *et al*., 2017). Soil communities have been previously used for biofilm studies (Burmølle *et al*, 2006; Burmølle et al., 2007; Ren *et al*., 2015 ; Johansen *et al.*, 2019). Soil Biodiversity is rich, with an estimated 10^{16} prokaryotic cells in just one tonne of soil (O'malley, 2014). However, diversity captured in laboratory conditions is thought to be considerably lower, some estimate only 1% (Amman, Ludwig and Schleifer, 1995), with new media and nutrient sources needed to capture currently unculturable soil microbes. Never-the-less, soil samples still provide high species diversity in laboratory cultures (Goldford *et al.,* 2018) and are rich in culturable pseudomonads (Ude *et al*., 2006). Soil microbial communities are appropriate for biofilm research as most soil bacteria are likely to be organised in biofilms on roots, litter or soil particles (Burmølle, Hansen and Sørensen, 2007), and a high percentage of soil microbes have demonstrated biofilm-formation at the A-L interface in static liquid microcosms (Ude *et al*., 2006). Within soil O_2 gradients stratify the environment, suggesting microbial soil communities will respond to conditions within static liquid microcosms and be subject to similar stratification (Fenchel & Finlay, 2008; Brune *et al.,* 2000). Stochastic and deterministic processes shape community composition and characteristics (Liu *et al*., 2019), changing community composition through species sorting or ecological filtering, and community function and traits as a response to environmental stratification. Bacteria incubated in static liquid microcosms establish a spatially structured environment through metabolic activity, creating an O_2 gradient, which will likely stratify complex microbial communities. Here, limiting O_2 becomes a selective pressure within the system.

O_2 gradients are ubiquitous in nature and are known to define microbial community function in fresh and sea water, and sediments and soils (Fenchel and Finlay, 2008; Brune *et al*., 2000). Within static liquid microcosms O_2 gradients are known to establish through the depletion of O_2 in the lower liquid regions, demonstrated in populations of *Pseudomonas fluorescens* SBW25 (Koza *et al*., 2011). This elicits and evolutionary response and Wrinkly Spreader mutants arise better able to exploit the ecological opportunity created by the high-O_2 region at the top of the liquid column through biofilm-formation (Koza *et al*., 2017). This is also found in other systems with strains of *Bacillus subtilis*, *P. aeruginosa* and *Shewanella oneidensis* (Kovács and Dragoš, 2019; Madsen *et al*., 2015; Yuan *et al*., 2013). Development of O_2 gradients through microbial metabolic activity is common throughout nature, often resulting in adaption to O_2 limiting conditions. O_2 gradients exist in soil (Noll *et al*., 2005) and iron-reducing bacteria involved in the corrosion of metal create anaerobic zones and an O_2 gradient through biofilm-formation on the metal surface (Angell, 1999). O_2

gradients also exist in the human body and human infection sites. In the lungs of cystic fibrosis patients infecting *P. aeruginosa* PA014 cells adapt to the hypoxic environment through the production of alginate and adaption to anaerobic respiration (Worlitzsch *et al.,* 2002), and O_2 concentrations have been shown to influence *P. aeruginosa* PA01 biofilm attachment (Skolimowski *et al.,* 2010). The gastrointestinal tract also subjects colonising bacteria with varying O_2 concentrations throughout, and cells require mechanisms for O_2 sensing which can trigger virulence and modulation of host response (Marteyn *et al*., 2010). Even within biofilms O_2 gradients exist, and can steepen with maturation (Stewart and Franklin, 2008). The effect of O_2 limitations in single-species studies clearly demonstrates a selective effect, requiring cells to adapt or evolve to the environmental conditions. As O_2 gradients are ubiquitous in nature, there is a need for single-species studies to be scaled-up to reflect the complexity of microbial communities and study a poly-microbial system response to limiting O_2 conditions.

Static liquid microcosms are spatially-structured upon incubation with microbial samples, as initial colonists act as ecosystem engineers or 'niche creators' by depleting the lower liquid region of O_2, creating a high-O_2 ecological niche at the top of the liquid column within the first three hours of incubation (Day, Laland and Odling-Smee, 2003; Steenackers *et al.,* 2016). The development of O_2 gradients in microcosms has been extensively studied in *P. fluorescens* SBW25 where a steep O_2 gradient is established. O_2 profiling revealed 0.1% of the normal dissolved O_2 below 1 mm after five hours and less than 1% of the normal dissolved O_2 below the top 200 µm after five days (Koza *et al*., 2011). This gradient is steepened through biofilm-formation at the A-L interface which prevents further O_2 from entering the system (Koza *et al.,* 2011; Loudon *et al*., 2016). In early growth stages the high-O_2 region represents an un-occupied niche in which strains capable of biofilm-formation or aerotaxis can access. However, the low-O_2 region consists of 90% of the liquid column (Koza *et al.,* 2011) and can support the growth of microaerobic or facultative anaerobic strains (Ferguson, Bertels and Rainey, 2013). Static liquid microcosms therefore provide a system to study the progression of A-L interface biofilm-forming communities, where a developing O_2 gradient likely stratifies complex communities, with biofilm-competent and aerotaxis strains competing for access to the high-O_2 region, and the liquid column supporting growth of microaerobic or facultative anaerobes. I hypothesise community succession within static liquid microcosms results in altered biofilm characteristics as a result of adaption and competition for the high-O_2 niche. This will also likely result in maximisation of community productivity, as members successful in competing for limiting resources

(nutrients) will increase in abundance, resulting in a lowering of species diversity and functionary redundancy.

Bacteria incubated in microcosm systems adjust behaviour to the surrounding environment, competing for limiting resources, and are shown to alter biofilm composition and structure (Nadell, Xavier and Foster, 2009). However, static liquid microcosms allow for a unique insight into life below the biofilm within the liquid column. Anti-biofilm therapeutics and biofilm reinforcement focusses on the biofilm community, however, biofilm communities come in contact with non-biofilm space. There is a knowledge gap surrounding the influence and interactions between biofilm communities and non-biofilm space. This non-biofilm space is likely to consist of planktonic cells, or small aggregates within liquid culture (Schleck *et al*., 2009). Static liquid microcosms provide a large liquid column supporting growth of planktonic cells under O_2 limiting conditions.

Within this chapter I aim to explore the effect of heterogenous O_2 limiting conditions on community aggregated and individual-strain-level traits of A-L interface biofilm-forming communities. Using a trait-based approach I will explore changes in community productivity, biofilm characteristics and phenotypic traits. Trait-based approaches describe individuals based on key traits, rather than species identity, where an individual can be identified by a combination of traits eluding to changes in structure and function of a community (Kiørboe, Visser and Andersen, 2018). I will use a serial-transfer approach to conduct short term ecological and evolutionary relevant experiments in static liquid microcosms where O_2 gradients establish. Transfers will introduce disturbance and fluctuation to the environment, where new microcosms introduce fresh nutrients and O_2 and prolonged incubation periods results in resource heterogeneity. To access the high-O_2 region within the system strains will require an appropriate colonisation strategy, and likely interact and compete for access. A soil-wash inoculum will provide an ecologically relevant model community, as communities of soil bacteria have been use in previous biofilm model studies (Tan *et al.,* 2017), and are likely to consist of aerobic A-L interface biofilm-competent pseudomonads (Ude *et al*., 2006). I aim to measure the successional and selective changes by using optical density measurements (OD_{600}) to indicate changes in productivity (Fiegna *et al.,* 2015). Optical density has been shown to be a robust measure of biomass and is directly comparable across microcosms and best reflects changes in productivity (Awasthi *et al*., 2016). However, it is important to note that cell shape, size and some extracellular polymeric substances and other light-scattering components can affect OD_{600} measurements (Myers, Curtis and Curtis, 2013). Changes in productivity will be measured in both the biofilm and

liquid column by using the cell localisation assay developed in Chapter 3. By further development of the localisation assay I hope to demonstrate community stratification and explore the influence of the liquid column community on the A-L interface biofilm and overall community productivity. Further changes in community-aggregated traits will be measured using the combined biofilm assay (CBA, Robertson *et al*., 2013) to determine changes in biofilm characteristics at both the community and isolate level, and a series phenotypic assays to identify traits of isolates within communities. As interactions are a key ecological dynamics and influence emergent community function I also intend to test for competitive and synergistic interactions, through spot-on-lawn based interaction assays (Schillinger and Lucke, 1989) and increasing productivity in co-culture compared to mono-culture indicating synergy, respectively.

Using data from the biofilm and phenotype experiments I aim to determine changes in community trait diversity utilising species indices to further evidence selection. This can be utilised to explore the relationship between diversity and productivity, and community resistance in A-L interface biofilm-forming communities. Community productivity has a complex relationship with species diversity and richness. Positive, negative and unimodal relationship have been found between community productivity and community diversity and richness (Mittelbach *et al.*, 2001; Smith, 2007). In communities of *P. fluorescens* SBW25 a unimodal relationship between diversity and productivity was found, however only in static heterogeneous environments and not under shaking conditions (Kassen *et al*., 2000). This suggests different spatial scales also influence this complex relationship. Similarly, species interactions also influence community productivity, where less negative interactions evolving overtime result in an increase in community productivity with higher diversity (Fiegna *et al*., 2015), demonstrated in communities of aquatic bacteria (Foster and Bell, 2012). Finally, community invasability (resistance of invading species, Jiang and Morin, 2004) will be explored. Community resistance also has a complex relationship with diversity, productivity and interactions. The insurance hypothesis suggests that more diverse communities are likely to contain taka with an appropriate trait allowing for increase community resilience, resistance and stability (Yachi and Loreau, 1999). This suggests a more diverse community is more resistant to community invasion, a finding in aquatic bacterial community studies (Jiang and Morin, 2004), and more functional redundancy within diverse communities can also increase community resistance (Allison and Martiny, 2008). However, other studies suggest that community interactions are more important in dealing with biotic stressors, making the community more resistant to invasion (BurmØlle *et al*., 2006).

4.1.1 Chapter Research Aims

The aim of this chapter is to develop a microcosm model system to explore the effects of an O_2 limiting environment and the occurrence of pulse disturbances on the productivity and community aggregated traits of ail-liquid interface biofilm-forming communities. This will explore the eco-evolutionary dynamics shaping the succession of bacterial strains within a serially-transferred community in response to an O_2 gradient. Serial-transfer of the model community will either favour biofilm–formation or not (i.e. static and longer incubations vs. shaken and shorter incubations), and isolates from the initial and final community will be selected for further strain-level analysis, to determine if changes in community properties are reflected in individual community members.

4.1.2 Research Objectives

1. Develop a microcosm system to study selection within multi-species biofilm-forming communities.
    I. Develop a soil-wash inoculum that can be stored and suitable for culture in static liquid microcosms.
    II. Demonstrate establishment of O_2 gradient in soil-wash inoculated static liquid microcosms.
    III. Confirm selective effect of changing incubation conditions and nutrient availability in soil-wash inoculated microcosms.
2. Explore the effect of selection in heterogenous O_2 limiting conditions on community-aggregated and individual strain-levels straits of A-L interface biofilm-forming communities.
    I. Design short-term evolution experiments using a serial-transfer approach.
    II. Explore community changes before and after selection using a trait-based approach to compare productivity, biofilm characteristics, and phenotypic changes at the community and individual strain level.
    III. Determine changes in species diversity and explore the relationship between diversity and community productivity.
    IV. Explore the presence and effect of species interactions on community productivity and selective changes.
3. Investigate the influence and interaction between coalescing biofilm and non-biofilm space.

I. Explore changes in productivity in the liquid column as a result of selection with heterogenous O_2 limiting conditions.

II. Determine the ability of community isolates to migrate between the biofilm community and liquid column and evaluate the importance of phenotypic plasticity.

4. Explore the resistance of selected communities to invading species.

I. Create neutrally marked strains with varying biofilm-forming capabilities from the *P. fluorescens* SBW25 lineage to act as invading strains.

II. Investigate the invasability of communities before and after selection utilising pairwise competitive fitness experiments.

III. Explore competitive interactions between the community members and invading species to determine the importance of competitive interactions in community invasability.

4.2 Results

4.2.1 Evidence of selection within microcosms system and experimental design

To investigate the impact of O_2 availability and time limitations between disturbance on selection within multi-species biofilm-forming communities, a viable model community and system is needed. The soil community selected must be viable for storage at -80˚C, and suitable for direct inoculation into microcosms. With the *Pseudomonas fluorescens* SBW25 system an O_2 gradient is rapidly established within the liquid column of static liquid microcosms, with 0.1% the normal dissolved O_2 below 1 mm after five hours (Koza *et al*., 2011). It was expected that O_2 gradients also establish in community static liquid microcosms, but to confirm a redox indicator dye was used to visualise the O_2 gradient. Incubation conditions within the microcosms system were tested with the soil-wash community to evidence selection, based on changes in community productivity (OD_{600}). This compared productivity with and without the presence of an O_2 gradient (static and shaken) and incubation time. Media type was also explored, as research suggests the highest level of diversity should be included in the set-up of a model system (Røder, Sørensen and Burmølle, 2016). From these results, several serial transfer experiments were designed to investigate the effect of time and presence of an O_2 gradient on selection within A-L interface biofilm-forming communities.

From interpretation of extensive literature, a common model bacteria community suitable for biofilm-based experimental systems are soil microbial communities. Soil samples were taken from an allotment in Dundee and a soil-wash was created which could be stored at -80˚C, and directly inoculated into microcosms to produce overnight inoculum for experiments. A small loss in diversity after freezing was expected, but hypothesised prolonged freezing would have no significant effect on the soil community. Samples were plated from the initial soil-wash, and after one week, three weeks and three months after storage and visually assessment of diversity was made. KB* agar plates produced the most visual diversity based on colony morphology, and very little changes were observed prior to and after storage (Figure 4.1).

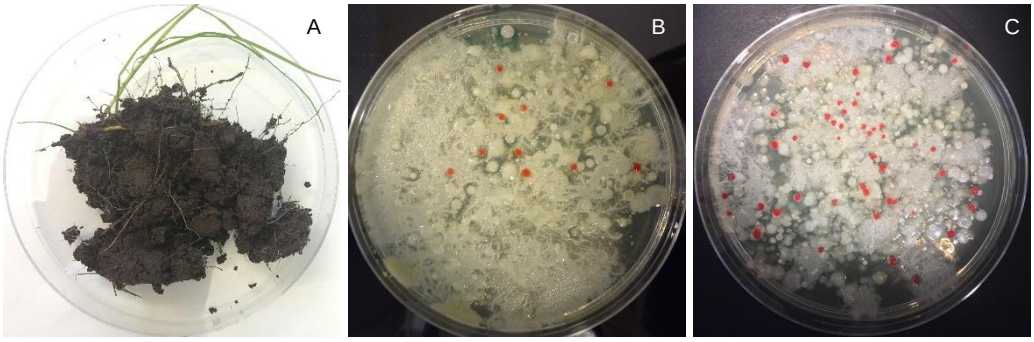


Figure 4.1. The initial soil sample and descriptive pictures of soil-wash community diversity before and after storage at -80˚C. Soil was collected from an allotment in Dundee, Scotland (A) and a soil-wash inoculum was created. The visual diversity was high from the initial soil-wash sample on KB* plates (B). Very little observable difference in diversity was seen after three months storage at -80˚C (C).

It was important to demonstrate that an O_2 gradient was established upon static incubation of the soil-wash community. In Chapter 3 the redox indicator methylene blue was utilised to confirm the O_2 gradient established within the *P. fluorescens* SBW25 system (Figure 3.3, E), where the liquid column turned blue to colourless as a result of O_2 depletion. A similar colour change was expected in soil-wash inoculated microcosms, as soil samples contain fast-growing aerobic pseudomonads (Ude *et al.*, 2006). The redox indicator was reduced within the liquid column, and evidence of reduction was present after 1 h of static incubation (Figure 4.2). After 6 h, only at the top of the liquid column below the A-L interface remained blue, indicating O_2 was depleted from most of the liquid column. After 24 h the soil community had established an A-L interface biofilm, preventing any O_2 from entering the system and no blue colour was observed. Once this sample was vortexed, the liquid rapidly became oxygenated, indicated by the blue colour re-appearing. This provides sufficient evidence of an O_2 gradient being established in soil-wash inoculated static liquid microcosms.

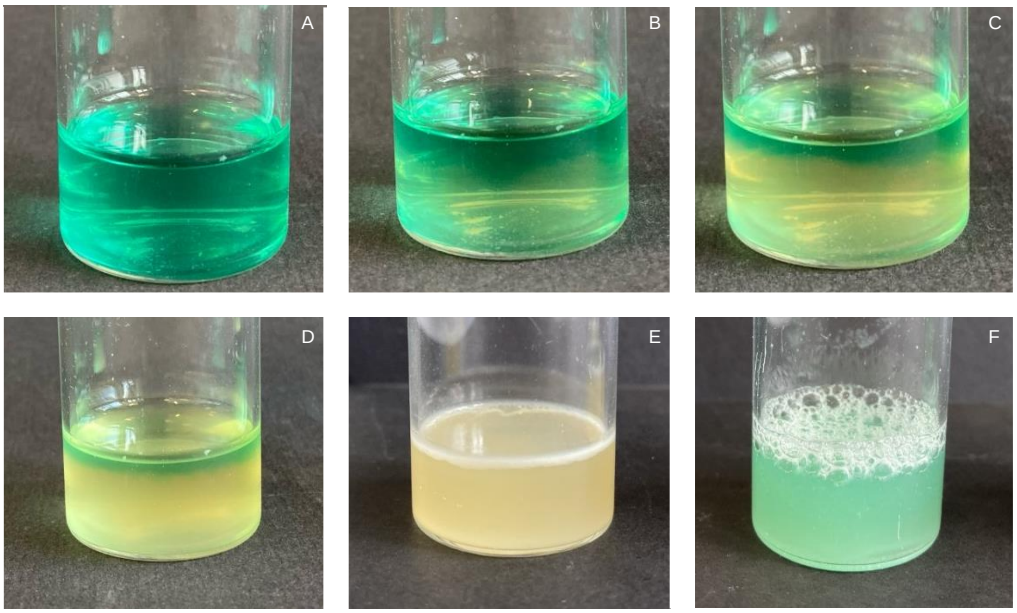


Figure 4.2. Establishment of an O_2 gradient in soil-wash inoculated microcosms over 24 h static incubation. The redox indicator methylene blue was added to static liquid microcosms inoculated with the soil-wash community. Methylene blue turns KB* media blue (A), but turns colourless as it is reduced, indicating O_2 depletion. The lower liquid region became depleted of O_2 after 1 and 3 h (B and C). After 6 h most of the liquid column was deplete of O_2, within only the top of the liquid column directly below the A-L interface still containing O_2 (D). After 24 h a biofilm at the A-L interface was established, and the entire liquid column was depleted of O_2 (E). O_2 was re-introduced into the liquid column by vortexing, and the liquid returned to the initial blue colour of the redox indicator (F).

With evidence of an O_2 gradient in static liquid soil-wash microcosms, initial tests could be made to explore the effect of O_2 availability, incubation period and media type on the growth of the soil-wash community. Here cell density (OD_{600}) is used as a measure of community productivity (Fiegna *et al*., 2015), where a decrease in the community productivity would indicate a bigger selective effect. Under static conditions, O_2 depletion acts as a selective pressure unlike shaking conditions where O_2 is homogeneous. Therefore a decrease in productivity was decrease in productivity was hypothesised under static conditions. Incubation period would also expect to alter community productivity and follow a standard growth curve, until plateau is reached indicating the carrying capacity of the system. Further incubation may even see a drop in community productivity through cell death and competition for limiting resources. Finally, changing media composition within the system was also predicted to alter community productivity, with KB* having the least selective effect based on the initial morphology diversity observations.

Community productivity of the soil-wash inoculum was compared in three different media types (KB*, LB and M9 supplement with glucose), in both static and shaken conditions over 13 days. Productivity measurements were taken at days 1, 3, 6, 10 and 13. Incubation conditions, period and media type had a significant effect on community productivity (Figure 4.3). Productivity in KB* media was significantly higher than LB and M9-glucose respectively, suggesting KB* support the highest level of community diversity (community productivity; KB* 2.24 ± 0.33, LB 2.14 ± 0.27 and M9-glucose 1.52 ± 0.23, $OD_{600,}$ TK-HSD, α = 0.05). Data from all media types were pooled to compared incubation conditions, and shaking incubation produced significantly higher community productivity than static conditions (community productivity; shaking incubation 2.02 ± 0.40 and static incubation 1.91 ± 0.44, $OD_{600,}$ TK-HSD, α = 0.05). As KB* produced the highest community productivity, community productivity overtime was further explored (Figure 4.2, C). Productivity in both static and shaken conditions significantly increased between one and three days, after which productivity plateaued with no further significant increase (community productivity; one-day shaking 1.86± 0.04 and static 1.42 ± 0.15, three-day shaking 2.41 ± 0.04 and static 2.29 ± 0.08, $OD_{600,}$ TK-HSD, α = 0.05).

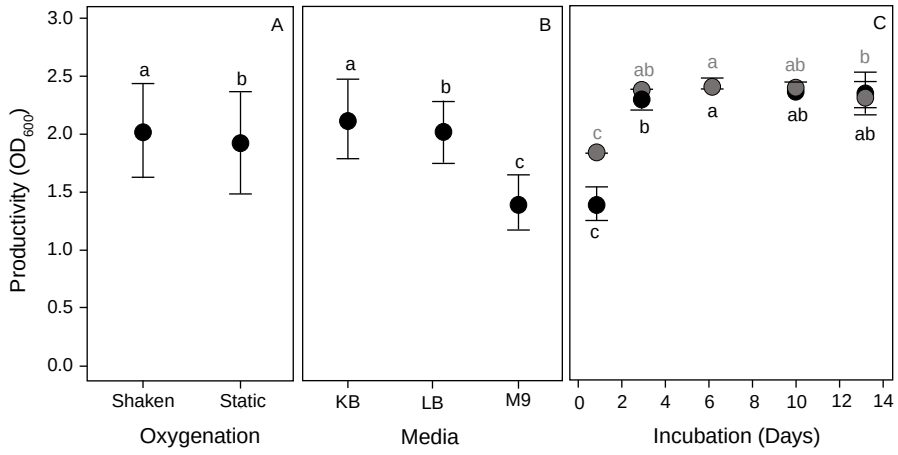
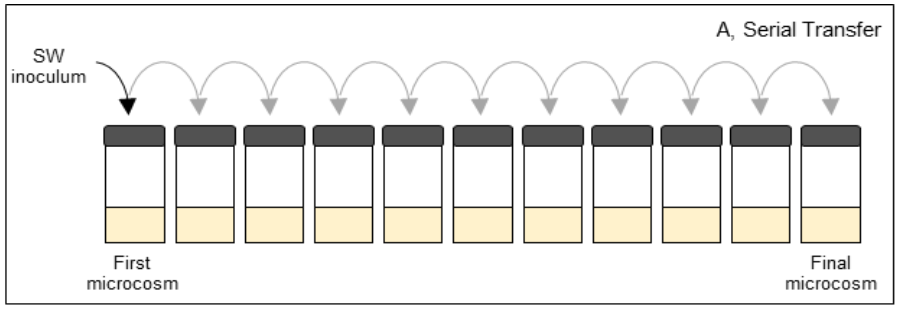
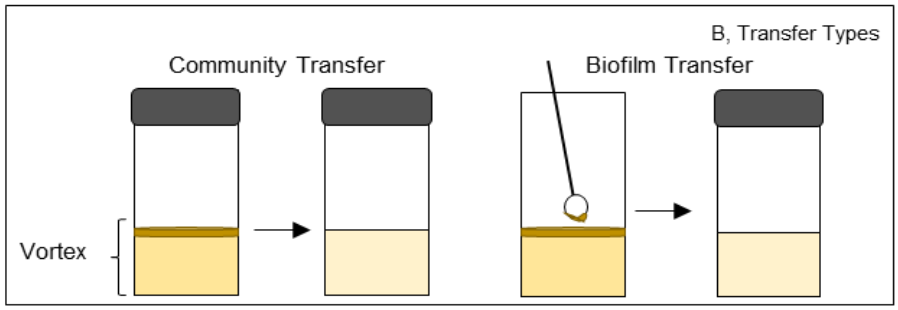
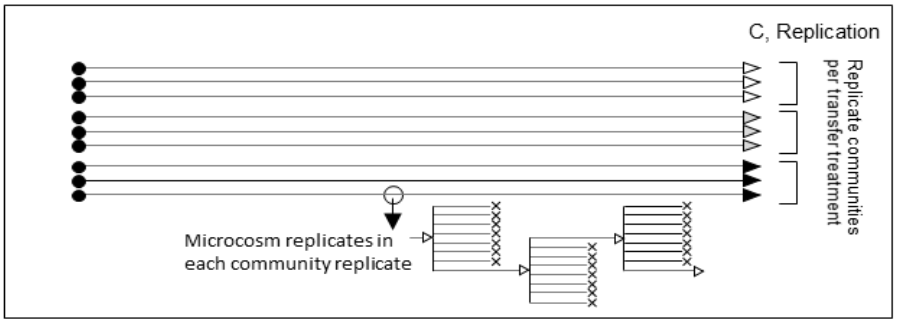


Figure 4.3. Altering conditions in microcosm system effects soil-wash inoculum productivity. Community productivity (OD_{600}) of the soil-wash inoculum was compared in different oxygenation conditions (static or shaken, Panel A) and media type (KB*, LB and M9 supplemented with glucose, Panel B) over 13 days. Panel C shows the progression of community productivity in KB* under static (black circles) and shaken conditions (grey circles) over the 13-day period, with productivity measured at day 1, 3, 6, 10 and 13. Means ± SE are shown. Means not linked by the same letter are significantly different (TK-HSD, α = 0.05).

From these results a serial-transfer experiment was designed (Figure 4.4). KB* media was used to provide a high starting biomass. One, three and six-day incubation periods were selected between transfers, providing three serial-transfer experiments with short, medium and long incubation periods. For each experiment, two transfer regimes were chosen where the whole community was transferred (vortexed prior to sampling) or only biofilm material was transferred, favouring selection for biofilm-formers. Both static and shaken conditions were conducted in community transfer experiments to compare selection both with and without an O_2 gradient. The biofilm transfer experiment was conducted under static conditions only to support biofilm-formation. The final-transfer communities produced by each of the treatments tested here are referred throughout out as the one, three and six-day ShC (shaken incubation with mixed community sample transfer), StC (static incubation with mixed-community sample transfer) and StB (static incubation with biofilm-only sample transfer) communities. For each serial-transfer treatment (n = 9) replicate communities were set (n = 3) and replicate microcosms were set (n = 8) for biofilm measurements (CBA) between each transfer. Each transfer 100 µl was transferred, representing 1.6% of the community. The serial-transfer experiments ran for a total of ten transfers, over 10 – 60 days, and each transfer seven replicate microcosms were measure using the CBA, and the final 8th replicate was used to transfer material to the next eight microcosms. Cumulative OD_{600} measurements were used to estimate the number of 'community' generations passed in each treatment of the serial-transfer experiment (Fiegna *et al*., 2015) and the effective population size estimated using a transfer inoculum of ~10^8 cells and 5 – 6 generations per transfer microcosm (Van den Bergh *et al.*, 2018).







D, Treatment Codes

Treatment	Incubation (Days)	Incubation Type	Transfer Regime
1-D StB	1	Static	Biofilm-only
1-D StC	1	Static	Entire Community
1-D ShC	1	Shaken	Entire Community
3-D StB	3	Static	Biofilm-only
3-D StC	3	Static	Entire Community
3-D ShC	3	Shaken	Entire Community
6-D StB	6	Static	Biofilm-only
6-D StC	6	Static	Entire Community
6-D ShC	6	Shaken	Entire Community

Figure 4.4. Serial-transfer experiment design. The serial-transfer experiments start with the soil-wash inoculum and were serially-transferred ten times (A). Each transfer the entire community was transferred, where microcosms were vigorously mixed, or the biofilm only was transferred using a wire loop (B). For each transfer experiment three replicate communities were set, and within eight replicate microcosms were set in which one was used for the next transfer and the others were measured using the combined biofilm assay (C). The treatments were 1-D, 3-D & 6-D (one, three and six-day)

ShC (shaken incubations with mixed-community sample transfer), StC (static incubations with mixed-community sample transfer), and StB (static incubations with biofilm-only sample transfer) (D).

4.2.2 Changes in community productivity and biofilm characteristics during serial-transfer of soil-wash community

From initial-selection experiments a large serial-transfer experiment was designed with an aim of measuring the effect of an O_2 gradient and time limitations on the community productivity, A-L interface biofilm characteristics and phenotypic traits of a multi-species community. To measure changes in productivity and biofilm characteristics the combined biofilm assay was used between each transfer. This allowed for a direct comparison between the initial soil-wash community and final transfer communities after serial-transfer to compare the effects of each transfer regime. Community productivity was expected to increase as the communities undergo change to fit the conditions within the microcosm environment and compete for resources (Bachmann *et al*. 2016; Hodapp *et al.* 2019). Similarly, differences between shaken and static treatments were predicted, with static conditions showing lower overall productivity due to the selective effect of O_2 limitations. The CBA also measures biofilm attachment (A_{570}) and strength (g), and it was hypothesised these community aggregated traits would significantly change, with a longer incubation period and static conditions favouring biofilm formers, resulting in an increase in strength and attachment between the initial and final communities.

In the serial-transfer experiments static microcosms showed signs of biofilm-formation in every replicate community established within the system after each transfer. The productivity cycling was estimated between 0.05 – 2.3 OD_{600} during the incubation periods, and an estimate effective population size was 5 x 10^8 (Van den Bergh *et al*. 2001). It was also estimated from cumulative OD_{600} measurements that experiments saw between 49 – 56 community generations (Fiegna *et al.,* 2015). Initial analysis suggested that changes in community productivity had occurred between the initial soil-wash and final transfer communities. Trends in community productivity between the initial and final transfer (Figure 4.5) demonstrated that productivity was 1.3x higher in microcosms incubated for longer periods between transfers (one-day, 1.48 ± 0.02, three-days 1.93 ± 0.01 and six-days, 1.96 ± 0.02, $OD_{600,}$ $\alpha = 0.05$) and 1.1x higher in shaken conditions compared to static (shaken 1.89 ± 0.02; static 1.74 ± 0.02, $OD_{600,}$ $\alpha = 0.05$). Productivity increased in the one-day community transfer experiments between the initial and final transfer, with little changes found in the one-day static biofilm transfer. Surprising community productivity decreased in

the final community transfers compared to the initial transfer in the three and six-day experiments. Spearman's correlation of the full transfer data set found a negative correlations between productivity and transfer number for 17/27 of the community replicates (ρ = -0.3 – -0.15; P = 0.01 – 0.02). This required further investigation to determine why productivity decreased and will be addressed in the following section.

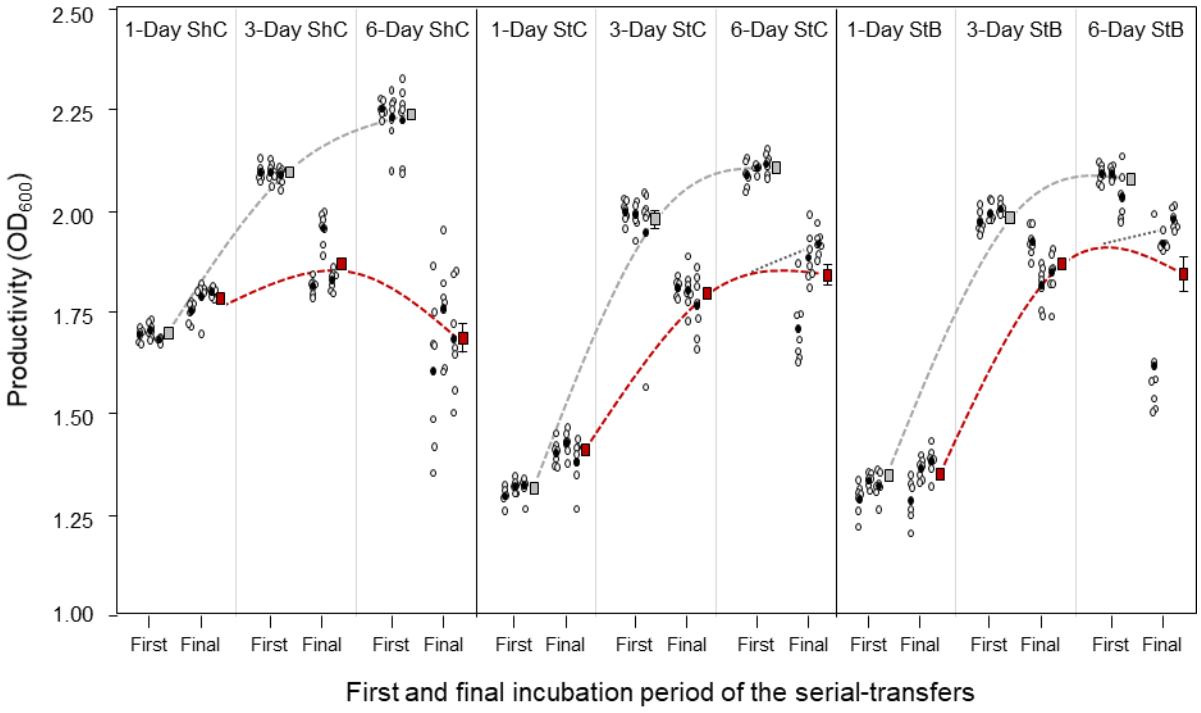


Figure 4.5. Changes in productivity between the first and final transfer microcosms in serial-transfer experiment. Community productivity (OD_{600}) was compared between the first (grey squares) and final (red squares) microcosms across the nine serial-transfer experiments. Median ± SE are shown, and replicate community data (black circles) and replicate microcosm data (white circles) is also included to shown where variation exists between replicates.

To identify the factors impacting community productivity (OD_{600}) across all transfer treatments, a modelling approach was used. Other approaches had been explored as data was not from the normal distribution, however upon recommendation and reflection from reviewers' comments about potential pseudo-replication issues, a mixed-effects (random) model was chosen. Microcosms replicate (n = 7) were nested within community replicate (n = 3) and were added as random effects to avoid issues with pseudo-replication. No outliers were removed, as variation was found between community replicates and there was no

reason to remove one rather than another, and divergence between replicate communities in experimental microcosms is common and not always a problem (Johansen *et al*., 2019). As a result, non-Normality was accepted within the distribution of residuals. Sample size was large, and there was no allowance for any loss in degrees of freedom and only high summary of fit values were accepted (*RSquared*), so robust models were produced. For confirmation, subsequent models were performed with outlier analysis until data conformed with normal distribution and can be found in Appendix 1.2.

Initial analysis suggested fluctuation of whole microcosms productivity, biofilm attachment (crystal violet A_{570}) and biofilm strength (maximum deformation mass, g) of replicate community microcosms throughout the transfer experiment (Appendix figures A1.1 – A1.3), so comparisons were made between the first and final transfer community for each treatment. Preliminary analysis of the initial and final microcosms data revealed significant differences in productivity between the first and final incubations across all transfer treatments. Modelling analysis revealed incubation period (one, three & six days), incubation conditions (shaken & static) and shift (from the first to the last incubation) had a significant effect on community productivity, but not sample type (community or biofilm) (See Table 4.1 for model summary). Community replicate was significant, however only accounted for 7.2% of the variation found in the model. Significant differences were found within effects and interactions, revealed using LS Means Differences Student's *t* and Tukey HSD (*a* = 0.05). Interactions included incubation period (1 day < 3 & 6 day; Q = 2.4), conditions (static < shaken), shift (final < initial), period x conditions (1 day/static < 1 day/shaken < 3 day/shaken < 6 day/shaken, 6 day/static & 3 day/shaken; Q = 2.9), period x sample type (1 day/biofilm & 1 day/mixed-community < 3 day/mixed-community, 6 day/biofilm, 6 day/mixed-community & 3 day/biofilm; Q = 2.8). This confirms significant changes found in community productivity between the first and final transfers in the serial-transfer experiment, as a result of changes in incubation period and incubation conditions, but not sample transfer type. The conformation model with outlier analysis can be found in Appendix 1.

Table 4.1. Summary of General mixed (random) models for changes in community productivity between first and final microcosm.

GLMM
RSquared
= 0.9

Fixed Effects	*P* - Value	F	**Interactions**	*P* - Value	F	**Random Effects**	% Variance
Incubation period (days)	0.000	$F_{2.2}$ = 628	Incubation period * initial or final microcosm	0.000	$F_{2.2}$ = 178	Community replicate	7.23
Initial and final microcosm	0.000	$F_{1.1}$ = 284	Incubation period * incubation conditions	0.000	$F_{2.2}$ = 109	Microcosm replicate [community replicate]	0
Incubation conditions	0.000	$F_{1.1}$ = 188	Incubation period * sample type	0.03	$F_{2.2}$ = 4		
Sample type	0.90	$F_{1.1}$ = 0.01					

As the combined biofilm assay was used throughout the serial-transfer experiment, changes in biofilm attachment (A_{570}) and biofilm strength (g) were also found. A mixed effects model was conducted utilising all data (productivity, attachment and strength) for initial and final microcosm comparison (see Table 4.2 for model summary). Modelling analysis revealed biofilm attachment, but not biofilm strength was significant. Community replicate was significant and accounted for 5.6% of the variation found in the model. Significant differences were found within effects and interactions, revealed using LS Means Differences Student's t and Tukey HSD (α = 0.05). Interactions included incubation period (1 day < 6 day < 3 day; Q = 2.3), conditions (static < shaken), shift (final < initial), incubation period x attachment (1 day/static < 1 & 6 day/shaken and 3 & 6 day/static < 3 day/shaken; Q = 2.8) and incubation period and sample type (1 day/biofilm & 1 day/mixed-community < 3 day/mixed-community, 6 day/biofilm, 6 day/mixed-community & 3 day/biofilm; Q = 2.8). The conformation model with outlier analysis can be found in Appendix 1.2. Across all static incubations Spearman's correlation analysis revealed a positive correlation between productivity and strength (ρ = 0.36, P = 0.01) and productivity and attachment (ρ = 0.25, P = 0.01). Strong biofilms were also well-attached structures (ρ = 0.45, P = 0.01), and both productivity and biofilm-formation

also increased with incubation period (incubation period and productivity, ρ = 0.67, strength, ρ = 0.65, and attachment, ρ = 0.49; P = 0.01).

Table 4.2. Summary of General mixed (random) models for changes in community productivity with attachment and strength biofilm data.

GLMM *RSquared* = 0.8

Fixed Effects	*P* - Value	F	**Interactions**	*P* - Value	F	**Random Effects**	% Variance
Incubation period (days)	0.000	$F_{2.2}$ = 33	Incubation period * incubation conditions	0.000	$F_{2.2}$ = 55	Community replicate	5.6
Initial and final microcosm	0.000	$F_{1.1}$ = 56	Incubation period * sample type	0.12	$F_{2.2}$ = 2	Microcosms replicate [community replicate]	0.0
Incubation conditions	0.000	$F_{1.1}$ = 73	Incubation period * Attachment	0.0007	$F_{2.2}$ = 7		
Sample type	0.98	$F_{1.1}$ = 0.0005	Incubation period * Strength	0.01	$F_{2.2}$ = 4		
Attachment	0.002	$F_{1.1}$ = 9	Attachment* Strength	0.84	$F_{1.1}$ = 0.04		
Strength	0.02	$F_{1.1}$ = 5					

As changes in biofilm-associated traits and productivity were apparent from modelling analysis, changes in biofilm-associated traits (biofilm attachment and strength) were further explored using the median data for each community (data not normally distributed, P < 0.05) and a non-parametric approach. This summarised changes in biofilm attachment (A_{570}) and biofilm strength (g) from the initial and final microcosms, measured through the combined biofilms assay. There were significant differences in biofilm attachment (A_{570}) and biofilm strength (g) between the initial and final transfers across the transfer communities (Figure 4.6, K-W Wilcoxon method, α = 0.05). There was a significant increase in attachment across

all transfer treatments, except from the one-day static biofilm transfer where a significant decrease was found, and in the six-day shaken transfer where no significant differences where observed. There was no significant difference in strength between the initial and final transfers of all shaken community transfers (one, three & six days). There was a significant increase in strength between the static community and static biofilm transfers in all experiments (one, three & six days). This confirms that biofilm-associated traits are also affected as a result of changes in incubation period and incubation conditions, but not sample transfer type.

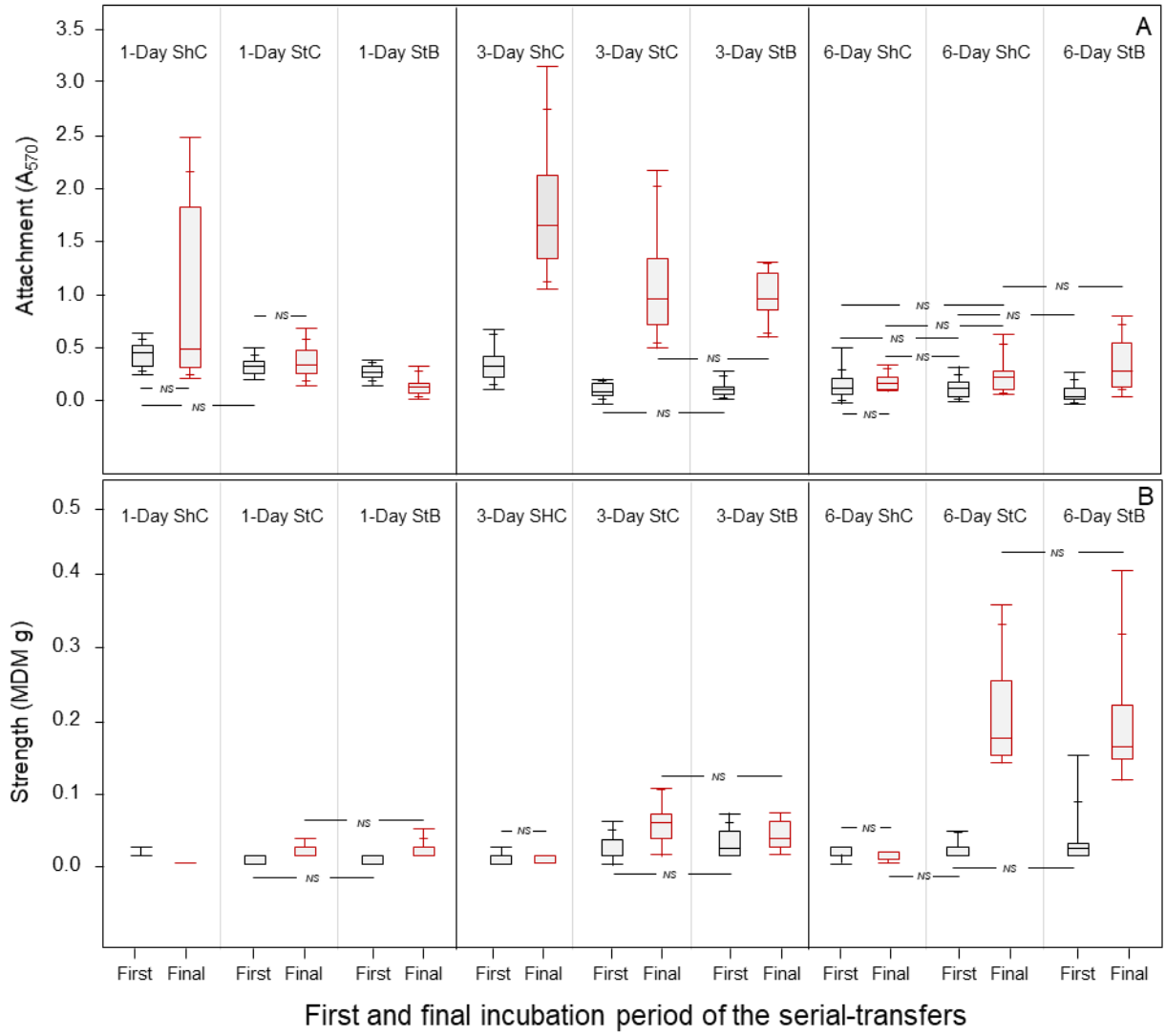


Figure 4.6. Summary of changes in biofilm attachment and biofilm strength between the first and final transfer microcosms in serial-transfer experiment. Community biofilm attachment (A_{570}) and biofilm strength (grams) was compared between the initial and final microcosms across all serial-

transfer experiments. Boxplots of the combined data for each community are shown. Significant differences are found between the initial and final community unless indicates with *NS* to show non-significant differences (K-W, Wilcoxon, α = 0.05).

Further analysis of the biofilm characteristics data was performed with principal component analysis, which was utilised to look at changes between the initial and final transfer microcosms utilising all measurements from the CBA (productivity, attachment and strength). The PCA demonstrates changes in biofilm-associated changes based on the incubation period between the transfer (one, three & six days, Figure 4.7). Changes are observed in the one-day transfer communities when comparing the initial and final microcosms community, with a small shift towards strength in both static transfer regimes, and a shift towards attachment and away from strength in the shaken community transfer. In the three-day transfer experiments, all transfer regimes demonstrate a clear shift away from growth and movement towards attachment. With a prolonged period of six-days, both static transfer regimes shift towards strength and attachment form the initial to final transfer microcosms, and the shaken regime shifts towards growth. This PCA analysis confirms community biofilm characteristics change as a result of changes in incubation period between transfer and incubation type (shaken or static).

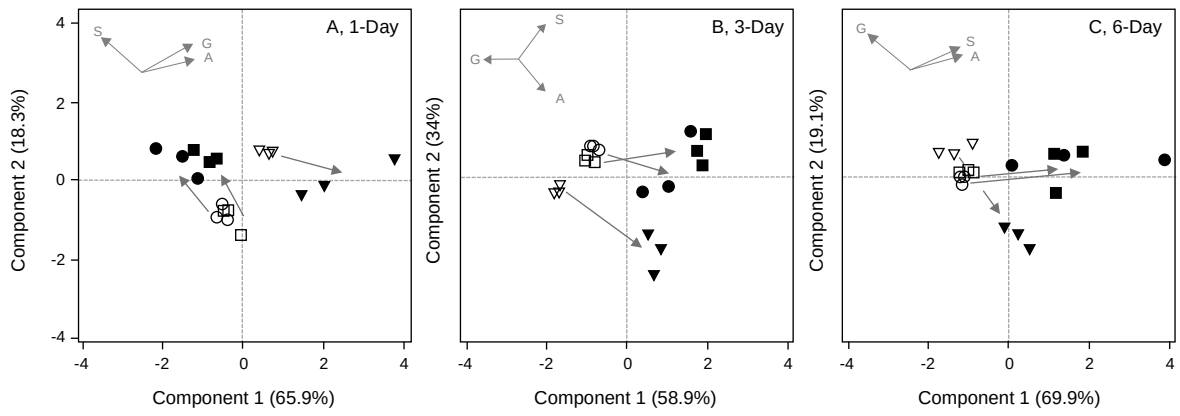


Figure 4.7. Principal component analysis at community-level biofilms shows changes in biofilm characteristics across transfer regimes and transfer incubation periods. Principal component analysis shows changes in growth (G, OD_{600}), attachment (A, A_{570}) and strength (S, grams) in community biofilm characteristics based on the transfer incubation period (Panel A = one-day, Panel B = three-day and Panel C = six-day) which shows changes in biofilm characteristics from the first transfer to the final transfer of the static biofilm (circles), static community (squares) and shaken community (triangles) transfer regimes. The shift in biofilm characteristics from the first

transfer to the final transfer are indicated with arrows. Panel A, one-day transfer experiments PC 1 (x-axis), 65.9 %; PC 2 (y-axis), 18.3%, Panel B, three- day transfer experiments PC 1 (x-axis), 58.9 %; PC 2 (y-axis), 34% and Panel C, six-day transfer experiments PC 1 (x-axis), 69.9 %; PC 2 (y-axis), 19.1%, (PC 3 is not significant based on the Eigen values for each PCA).

4.2.3 Decrease in community productivity with prolonged incubation is a result of the accumulation of toxic waste products, nutrient depletion and negative interactions

Within the serial-transfer experiment one-day transfer communities increased in community productivity as expected, suggesting adaption of community members to conditions within the microcosm system. However, productivity in three and six-day serial-transfer communities decreased, suggesting further investigation is needed to determine why community productivity is failing to maximise within these communities. Bacteria constantly modify the surrounding environment through metabolism (McNally and Brown, 2015), resulting in the depletion of nutrients and accumulation of toxic waste products. As nutrients becomes limiting within the system, microbial communities are likely to compete for resources through active secretion of toxic compounds, or adaption in substrate consumption (Studdendiek, Vargas-Baustista and Straight, 2016). The initial soil-wash community and six-day transfer communities were utilised to determine if environmental modification through the depletion of nutrients and build-up of toxic waste products and secondary metabolites restrained community productivity. In addition, the presence of competitive phenotypes or missing beneficiaries lost during transfer may also explain the reduction in productivity. Interactions assays were used to investigate the presence of positive and negative interactions. It was hypothesised both the accumulation of waste products and depletion of nutrients result in reduced community productivity, and increased competitive phenotypes are found within six-day communities contributing to the fall in productivity. To investigate the toxic effect of an aging community environment, aged media (spent media with prolonged growth period allowing for nutrient depletion, and waste product and metabolite accumulation) was created by incubating the soil-wash community and the six-day biofilm community for one, three and six days before removing cells. Fresh community samples were inoculated in aged media and fresh KB*, and productivity (OD_{600}) was measured after three days. If the aged media contained toxic waste products, or insufficient nutrients a decrease in productivity would be expected. The model bacterium *P. fluorescens* SBW25 was also used to test the toxic effect of aging media.

There was a significant difference in productivity in aged media compared to fresh KB* in all three test communities and populations (Figure 4.8, A). There was no significant difference between one-day aged media and KB*. There was a small but not significant difference in productivity of the six-day biofilm community and *P. fluorescens* SBW25 in media aged for three-days, and a significant difference in productivity in the initial soil-wash community in three-day old media compared to KB* media (SW community productivity; fresh KB* 1.67 ± 0.005, three-day media 1.26 ± 1.27 OD_{600}, TK-HSD, α = 0.05). There was a significant decrease in productivity across all samples when incubated in media aged for six-days compared to fresh KB* (SW community productivity fresh KB* 1.67 ± 0.005 and six-day media 0.85 ± 0.03; six-day community productivity fresh KB* 1.67 ± 0.07 and six-day media 0.98 ± 0.03; *P. fluorescens* SBW25 productivity fresh KB* 1.36 ± 0.01 and six-day media 0.4 ± 0.02, OD_{600}, TK-HSD, α 0.05). This suggests a prolonged incubation period of six-days has sufficient nutrient depletion and toxic waste production to cause significant inhibition of *P. fluorescens* SBW25 growth, and possible community growth in transfer experiments.

A clear inhibitory effect of the six-day aged media was confirmed, however further investigation was required to determine if productivity inhibition was a result of nutrient depletion, toxic waste accumulation, or a combination of both. Fresh KB* samples were diluted six-day aged media or sterile water, and the productivity of *P. fluorescens* SBW25 was compared after three days. If productivity was inhibited by a combination of nutrient depletion and toxic waste accumulation, productivity of *P. fluorescens* SBW25 would expect to decrease more in KB* diluted with aged media, compared to KB* diluted with water only. There was a significant decrease in productivity in diluted KB* compared to standard KB*, with both aged media and water (Figure 4.8, B). At a KB* concentration of x0.5 and x0.2, productivity was significant lower in KB* mixed with aged media compared to water (KB* 1.74 ± 0.006; KB* x0.5 water 1.63 ± 0.003 and aged media 1.43 ± 0.01; KB* x0.2 water 1.34 ± 0.01 and aged media 1.25 ± 0.007, $OD_{600,}$ TK-HSD, α 0.05). When KB* concentration reached x0.1, productivity in KB* diluted with water was significantly lower than aged media (KB* x0.1 water 0.9 ± 0.02 and aged media 1.09 ± 0.02, $OD_{600,}$ TK-HSD, α 0.05). This data suggests that the inhibition of community productivity may be a combination of both nutrient depletion and toxic waste accumulation.

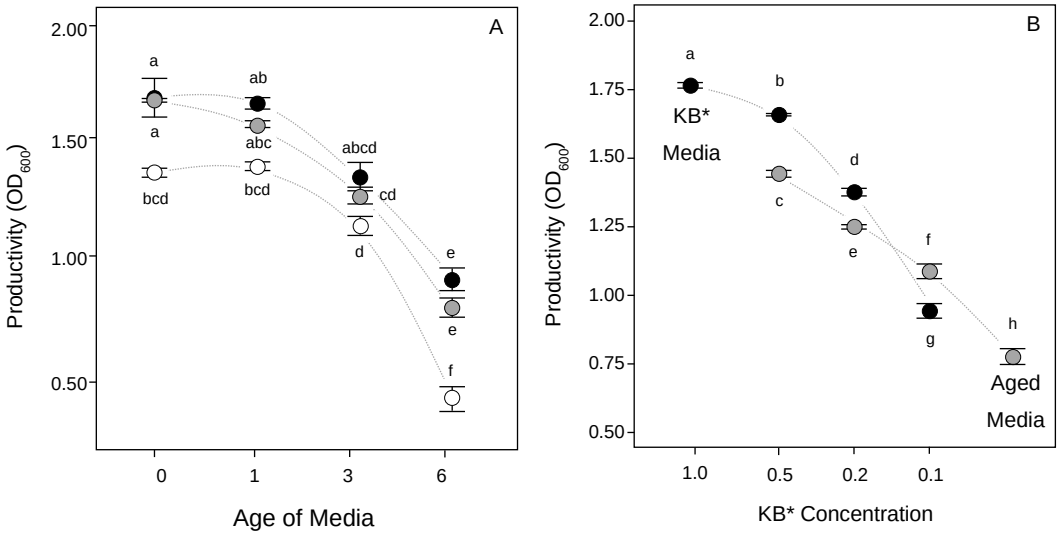


Figure 4.8. Toxic effect of aged media limits community productivity. Communities incubated for prolonged time periods (three and six days) result in the accumulation of metabolic waste products and depletion of nutrients which limit community productivity. As the age of the media increases (Panel A), the productivity of the initial soil-wash community (grey circles), six-day static biofilm community (black circles) and the model bacterium *P. fluorescens* SBW25 (white circles) significant decrease. The productivity of *P. fluorescens* SBW25 was compared in shaken microcosms with KB* diluted with water (black circles) or aged media (grey circles, Panel B) and productivity is limited in both. Mean ± SE (n = 5) are shown. Means not linked by the same letter are significantly different (TK-HSD, *a* = 0.05). Trend lines are descriptive only. This data has been used in the Jerdan et al. (2020) publication.

Competitive and antagonistic interactions are common in biofilm-forming communities, and negative interactions through interreference competition can inhibit community productivity. To indicate the presence of species that capable of producing competitive or antagonistic secondary metabolites a spot-on-lawn approach was used. Isolates (n = 24) from the initial soil-wash were plated as lawns, and isolates from the six-day transfer communities (n = 12 from each community) were spotted on top. Competitive interactions were classed as reduced growth by the lawn strain near the interface with the spot colony. Antagonistic interactions were classed as an inhibition zone of the lawn strain around the spot colony.

Competitive and antagonistic interactions were found in all of the six-day transfer communities (Figure 4.9) and a Chi-square test of independence confirmed that the origin of the spot isolate and the interaction type were dependent (*ChSquared*, $P < 0.001$). More competitive interactions were observed in both the six-day static communities compared to the six-day shaken community (6-D StB = 56, 6-D StC = 62 and 6-D ShC = 24, competitive

interactions) and more antagonistic interactions were observed in the six-day static biofilm community (6-D StB = 56, 6-D StC = 1 and 6-D ShC = 1, antagonists interactions). Odds ratios were calculated and within the six-day static and six-day biofilm transfer communities it was ~4 times more likely to find isolates that produce negative interactions than no interactions ($P < 0.001$), with no significant differences between the static and biofilm communities found ($P = 0.29$). These results suggest that more community members present within the six-day static and six-day biofilm communities were capable of producing secondary metabolites compared to the six-day shaken community, which may result in increased growth inhibiting interactions within the community.

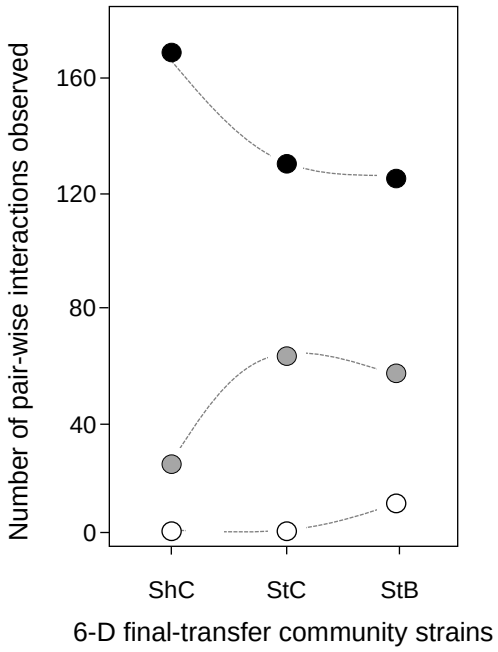


Figure 4.9. Competitive and antagonistic interactions were observed in the six-day biofilm communities. Competitive and antagonistic interaction were observed in the six-day final communities using a spot-on-lawn approach, where isolates from the six-day communities were spotted against isolates from the initial soil-wash community. All communities demonstrated competitive and antagonistic interactions, the six-day biofilm transfer having the highest number of antagonistic interactions. A Chi-square test of independence indicates that strain origin (ShC, StC & StB) and interaction type (antagonistic, competitive and neutral) were dependent (*ChiSquared*, $P < 0.001$). Trends lines are descriptive only. This data has been used in the Jerdan et al. (2020) publication.

As neutral interactions were more common within the six-day transfer communities, It was predicted that the productivity of static test microcosms inoculated with a mix of strains (n = 1, 2, 4 & 8) should not be significantly different from microcosms inoculated with the corresponding community sample. In addition, an increase in productivity in mixed strain microcosms compared to monoculture would suggest synergy between community members. Mixed community isolate experiments were initially conducted with the biofilm transfer communities. Here, eight isolates from a replicate community were randomly selected, and inoculate in combinations of 1, 2, 4 and 8 strains, and productivity (OD_{600}) was compared to the whole community sample. Results may also be influenced by the relationship between species-richness and productivity, a complex relationship in which positive, negative and unimodal relationships can be found (Smith, 2007). A unimodal relationship between productivity and richness was expected within the mixed community isolate tests, with neutral or synergistic interactions found in mixed strain microcosms of 2 – 8 strains increasing productivity compared to monoculture, and a decrease in productivity in the whole community sample through increased competition for resources.

In tests using the one and three-day static biofilm community isolates and community samples, no significant differences were observed (Dunnett's, $\alpha = 0.05$, comparing mixed isolate productivity to community productivity, Figure 4.10, A and B). However, a single isolate in the three-day biofilm community with very low productivity (OD_{600} = ~1) did not drop overall productivity when placed in co-culture, suggesting combinations of community isolates could have synergistic relationships. In the six-day biofilm community a significant decrease in productivity was found in the community sample compared to microcosms with combinations of 2, 4 and 8 strains ($P = 0.001$ - 0.009, Dunnett's, $\alpha = 0.05$. Figure 4.9, C). This may reflect the competitive interactions found in this community. Subsequent analysis was conducted with the six-day static community and shaken community, but no significant differences were found. These results confirm the complex network of competitive, synergistic and neutral interactions found in microbial communities. However, competitive phenotypes are more likely to be found when communities are incubated for a prolonged period (six-days) between transfers.

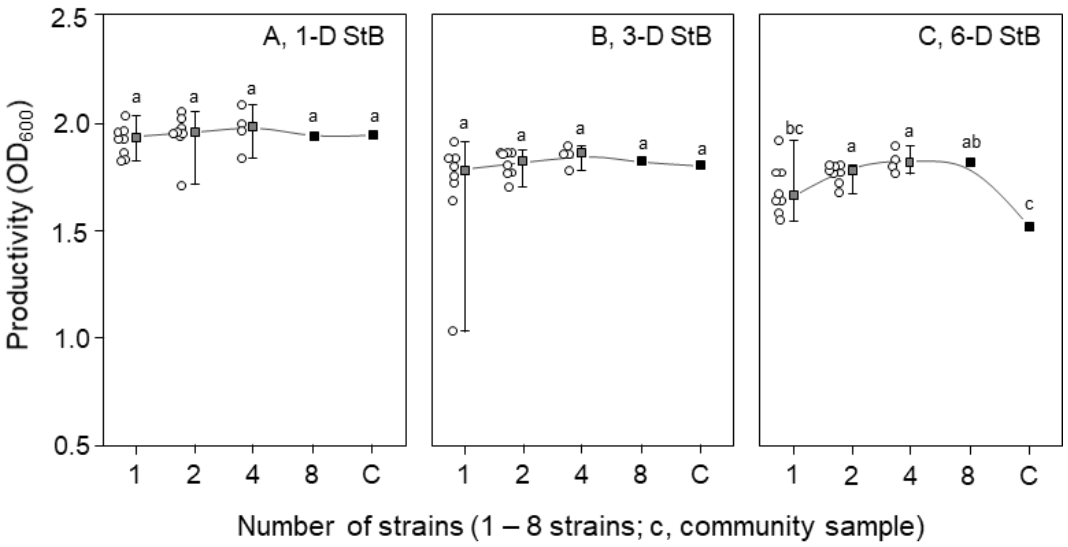


Figure 4.10. Comparison of mixed strain microcosms and overall community productivity. Productivity (OD_{600}) in mixed strain microcosms (strains n = 1, 2, 4 & 8) were compared against the corresponding community sample to investigate the dominance of neutral interactions within transfer communities. Median plus range is shown, medians not linked by the same letter are significantly different (Dunnett's method, *a* = 0.05). This data has been used in the Jerdan *et al*. (2020) publication.

4.2.4 Changes in community aggregates traits are reflected at the individual isolate-level and indicate changes in diversity

Across community transfer experiments there is evidence of community changes in productivity and biofilm-associated traits (attachment and strength). However isolate-level analysis is needed to fully capture changes caused by selection under O_2 limiting conditions. Individual isolates were selected for further analysis to explore changes at the individual isolate-level. Isolates from the initial soil-wash community and all nine end communities (across all three replicate communities) were chosen randomly and analysed using the combined biofilm assay and a series of phenotypic and behavioural assays. changes in community biofilm characteristics were expected to be reflected in individual isolate traits, with communities showing significant changes in biofilm characteristics also containing isolates that demonstrate similar changes. Changes in phenotypic and behavioural traits of isolates within the end communities were also expected, as individuals can succeed or adapt within a community through adapting behavioural or functional traits. From biofilm and phenotype data operational taxonomic units (OTU) were assigned to calculate trait-based species diversity, utilising species indices. Unique combination of microcosm productivity,

biofilm strength and biofilm attachment (very low, low, medium and high, based on CBA isolate data) were assigned as a biofilm-associated OTU, and a unique combination of phenotype traits were assigned as a phenotype-associated OTU. Here, a decrease in biofilm and phenotypic diversity in the final communities as a result of the selection pressures within the microcosm system were expected, with the biggest decrease in diversity predicted in the six-day static communities, where communities were subject to O_2 limitations for a prolonged period.

Initial observations of colony morphology in each final community suggests changes in diversity compared to the initial soil-wash community. Each community demonstrated differences in colony morphs, including variation in colony size and colour, and some communities demonstrated more mucoidal or fluorescent colonies (Figure 4.11, see Figure 4.1 for initial soil-wash plates). Isolates selected from each community (n = 8 isolates per replicate community to give n = 24 isolates per community treatment) were analysed using the combined biofilm assay measuring productivity (OD_{600}), attachment (A_{570}) and strength (maximum deformation mass, g). Isolates were then compared using eight phenotypic assays testing for surfactant production (drop collapsed assay and foam production), catalase and oxidase production, siderophore production, gram staining, pH (indication of pH above 7) and growth on *Pseudomonas* selective agar. Initial hierarchical cluster analysis of the biofilm and phenotypic data shows isolates grouped into 5 distinct clusters (Figure 4.12). A test of independence of biofilm and phenotype data confirmed strain clustering was dependent on the incubation period (one, three and six days, *ChiSquared*, $P < 0.001$) and transfer treatment (ShC, StC and StB, *ChiSquared* , $P < 0.001$), and the initial soil-wash strain were randomly spread across the tree (soil-wash isolates and all end isolates, *ChiSquared*, biofilm data, $P = 0.35$ and phenotype data $P = 0.52$).

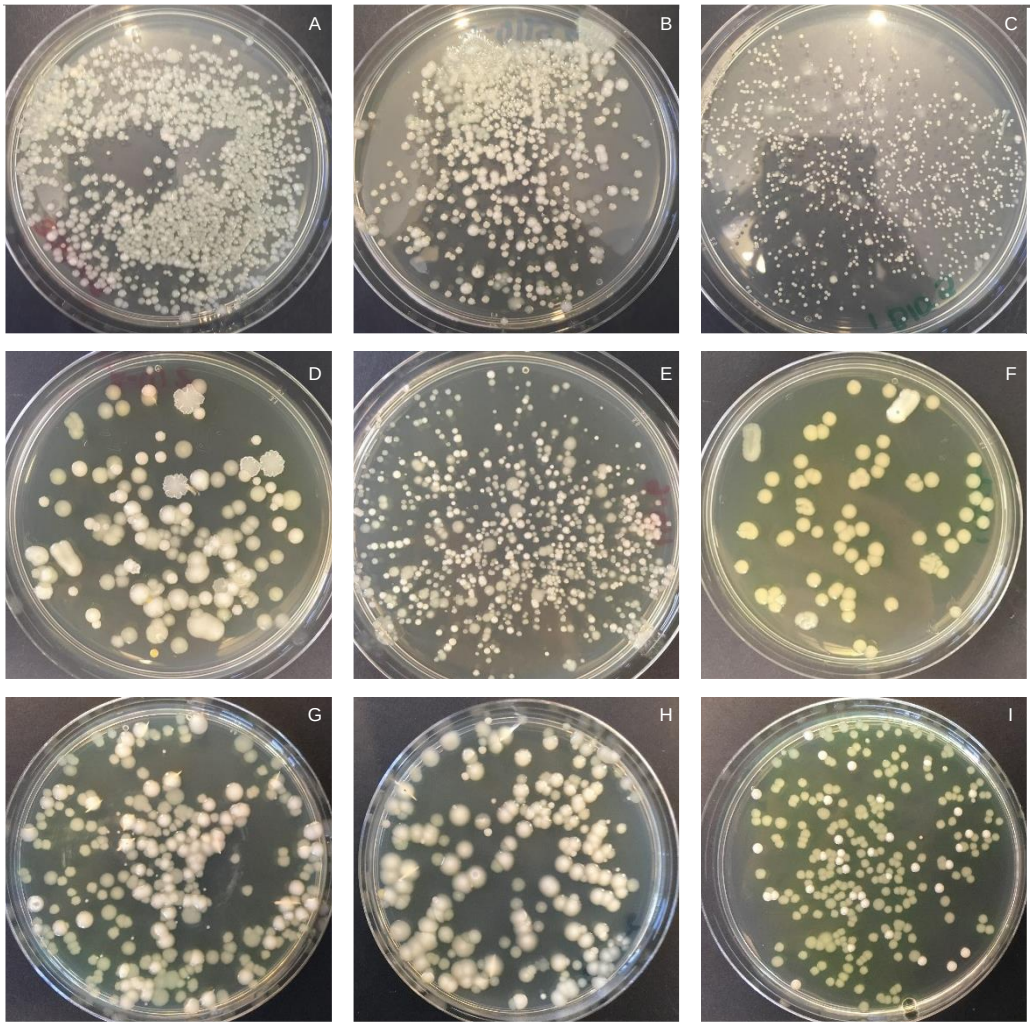


Figure 4.11. Changes in colony morphology of serial transferred communities. Communities undergone serial-transfer in microcosms demonstrate observable differences in colony morphology of community members, including size and colour. Shown are one replicate from each final transfer community (A = 1-D ShC, B = 3-D ShC, C = 6-D ShC, D = 1-D StC, E = 3-D StC, F = 6-D StC, G = 1-D StB, H = 3-D StB and I = 6-D StB). This figure is descriptive only.

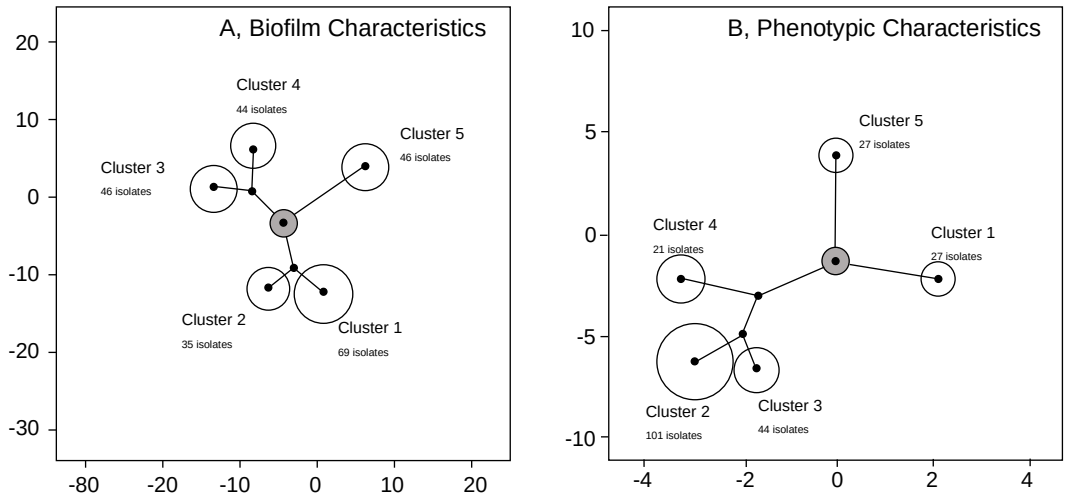


Figure 4.12. Hierarchical cluster analysis of biofilm and phenotype data of all community isolates. Isolates from the initial soil-wash and all end communities were compared using the combined biofilm assay (microcosms growth, OD_{600}; attachment A_{570} and strength, g) and a series of phenotypic assays (gram staining, oxidase and catalase production, siderophore production etc.). Hierarchical cluster analysis shows isolates are characterised into five clusters for both the biofilm data set (Panel A), and the phenotypic data set (Panel B). A Chi-squared test of independence indicates clustering was dependent of the transfer incubation period (1, 3 and 6 days) and the transfer treatment (ShC, StC & StB) and (*ChiSquared P* < 0.001), and the initial soil-wash isolates are randomly distributed across the tree (soil-wash isolates and all end isolates, biofilm data *ChiSquared*, *P* = 0.35 and phenotype data *P* = 0.52). The number of isolates in each cluster is indicated. This data has been used in the Jerdan *et al*. (2020) publication.

To investigate if changes in community-aggregated traits are reflected by changes in individual isolate biofilm characteristics, PCA was performed for the CBA data of all 240 isolates (Figure 4.13). There were little changes observed in one-day community isolates, with strong overlapping between the biofilm characteristics of the initial isolates and the one-day shaken community. This reflects the smaller changes in community biofilm characteristics found in community-level analysis. Further differentiation can be made in the three-day and six-day transfer community isolates. There was a significant shift in biofilm trait space in the six-day static community and static biofilm transfers, with a clear shift towards biofilm attachment and growth. This was not found in the six-day shaken transfer experiment, reflecting little changes in biofilm strength and attachments in community-level

analysis. This confirms significant differences in biofilm-associated traits at the isolate-level occur in the presence of an O_2 gradient during prolonged incubation periods.

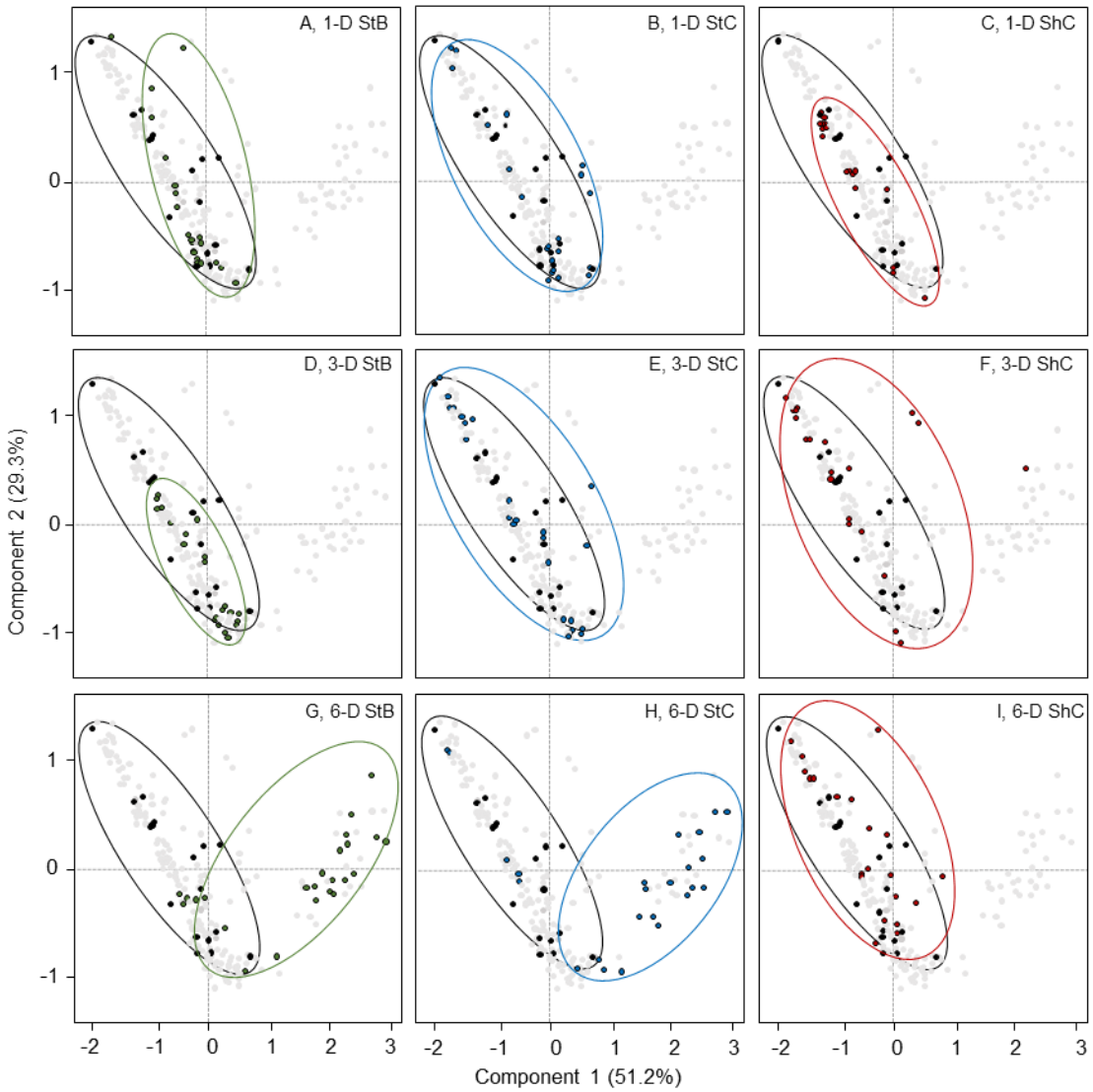


Figure 4.13. Principal component analysis at individual isolate-level reflects changes found at the community level. The growth (OD_{600}), attachment (A_{570}) and strength (g) of individual isolates from the initial and final communities (n = 24 per community) were also measured. The same principal component graph has been reproduced to highlight changes in community isolate biofilm characteristics from the initial soil-wash (highlighted in black in each panel, and each of the final transfer communities. PC 1 (x-axis), 51.2 %; PC 2 (y-axis), 29.3 % (PC 3 is not significant based on the Eigen values). This data has been used in the Jerdan *et al.* (2020) publication.

Biofilm-formation at the A-L interface was common amongst isolates before and after selection. The frequency of isolates capable of biofilm-formation was compared between the initial soil-wash and final transfer communities using the Odds ratio calculation (Table 4.3).

The number of biofilm-forming and non-biofilm forming isolates for each community were compared against the initial soil-wash (initial soil-wash has 15 of the 24 tested isolated capable of biofilm-formation). There were significantly more biofilm-forming isolates found in the one-day static biofilm transfer experiment, and in the six-day static biofilm and static community transfers, where it was 4 – 7x more likely to find biofilm-forming member in the six-day static transfers (*ChiSquared P* < 0.02 – 0.06).

Table 4.3. Odds ratio of biofilm-forming isolates within end transfer communities. This data has been used in the Jerdan *et al*. (2020) publication.

Isolates from (community)	Number of biofilm-formers	Odds ratio	*P* - Value
1-D ShC	12	0.6	0.38
1-D StC	19	2.3	0.21
1-D StB	24	30.0	0.02
3-D ShC	4	0.1	0.002
3-D StC	8	0.3	0.05
3-D StB	13	0.7	0.56
6-D ShC	15	1.0	1.0
6-D StC	21	4.2	0.06
6-D StB	22	6.6	0.03
Odds ratio calculated using the number of biofilm-forming and non-biofilm-forming SW strains. SW contained 15 out of 24 isolates that formed biofilms			

Phenotypic characteristics were recorded for all 240 isolates, and results were recorded as 'positive' or 'negative'. Table 4.4 demonstrates the Odds ratio for each phenotypic test for final transfer communities. Odds ratio was calculated using the number of positive results in comparison to the initial soil-wash for each assay. The drop collapsed assay and foam production indicate surfactant-like production, and both assays conclude that there are significantly less isolates producing surfactant-like products within the three and six-day shaken communities compared to the initial soil-wash (Odds ratio 0.12-0.2, *P* = 0.001 – 0.03). Both three-day static transfer communities also had significantly less isolates producing surfactant-like products. Within the six-day static transfers the drop collapse assay suggest significantly less isolates producing surfactant-like products (Odds ratio 0.12 – 0.13, *P* = 0.003) however, foam production results suggest significantly more isolates producing

surfactant-like products (Odds ratio 4.6, *P* = 0.09). This may be due to other excreted products from cells such as extracellular polysaccharides also producing similar results to surfactant production in the foam assay.

Table 4.4. Odds ratio of phenotype characteristics in final transfer communities.

Community Phenotype	**1-D ShC**	**3-D ShC**	**6-D ShC**	**1-D StC**	**3-D StC**	**6-D StC**	**1-D StB**	**3-D StB**	**6-D StB**
Drop Collapsed									
Odds ratio	0.12	0.12	0.13	1.5	0.27	0.12	0.78	0.12	0.13
P - value	0.03	0.03	0.03	0.26	0.06	0.03	0.36	0.03	0.03
Foam Production									
Odds ratio	0.6	0.14	0.2	1	0.14	4.6	0.76	0.08	4.6
P - value	0.23	0.002	0.009	0.5	0.002	0.093	0.35	0.0002	0.09
pH above 7									
Odds ratio	0.005	0.03	0.06	0.04	0.06	0.45	1	0.03	1
P - value	0.00009	0.001	0.007	0.001	0.007	0.26	0.5	0.001	0.5
Catalase Production									
Odds ratio	0.21	2.5	1	0.2	2.05	7	3	1.6	2.5
P - value	0.09	0.09	.05	0.08	0.15	0.002	0.05	0.7	0.09
Oxidase Production									
Odds ratio	7.85	3.57	0.604	0.51	0.49	2.71	1.19	0.6	2.71
P - value	0.007	0.03	0.19	0.12	0.12	0.06	0.38	0.19	0.06
Siderophore Production									
Odds ratio	0.23	0.1	0.05	0.23	0.48	8.27	0.23	1	8.27
P - value	0.01	0.004	0.003	0.01	0.11	0.002	0.01	0.5	0.002
Growth on PSA									
Odds ratio	2.18	2.18	0.34	2.09	2.09	2.18	2.18	2.09	2.18
P - value	0.26	0.26	0.11	0.27	0.27	0.26	0.26	0.27	0.26
Gram Negative									
Odds ratio	0.34	1.85	3.42	1	3.42	3.42	3.42	3.42	3.42
P - value	0.08	0.3	0.15	0.5	0.15	0.15	0.15	0.15	0.15

It was significantly less likely that isolates produced cultures above a pH of 7 within all shaken transfer experiments (Odd ratio 0.005 – 0.06, *P* = 0.001) and the one-day and three-day static community transfers (Odds ratio 0.04 – 0.06, *P* = 0.001 – 0.007). There was significant selection for isolates producing catalase and oxidase in the three-day shaken community, and the six-day static community and static biofilm transfers (Odds ratio 2.5 – 7. *P* = 0.002 – 0.09). The one-day shaken community also demonstrated significant selection for oxidase producers (Odds ratio 7.85, *P* = 0.007). It was 8x more likely to find isolates producing siderophores in the six-day static community and biofilm transfers compared to the initial soil-wash (Odds ratio 8.27, *P* = 0.002), and all shaken transfer communities had significantly fewer siderophore producing isolates (Odds Ratio 0.05 – 0.23, *P* = 0.003 – 0.01). There were no significant differences in the number of isolates that were capable of growth on *Pseudomonas* selective agar compared to the initial soil-wash. A similar results was found in the number of gram negative bacteria, as the initial soil-wash contained a high number of gram negative isolates, and this was retained throughout all transfer communities, with the three and six-day static community and static biofilm transfers containing no gram-positive isolates. Phenotypic and behavioural characterisation demonstrates changes between the initial soil-wash and final community, and differences between transfer regimes.

All data from the biofilm and phenotype experiments was utilised to analyse changes in community diversity. Isolate-level biofilm data from all 240 strains were pooled, and each measurement (growth, attachment and strength) was ordered from smallest to highest. Each data set was split into 4 sections using median and quartile range. This provided each isolate with a 3-digit code, and each code was used to represent a taxonomic unit. For the phenotype data, positive or negative results were converted to numeric values (positive = 1, negative = 2) and provided a 7-digit code for each isolate. The Simpson reciprocal (1/D) and Shannon index (H) species indices were used to calculate diversity for each community. In both indices, the higher the index value, the higher diversity within the community. A modelling approach confirmed there was a significant difference in diversity between communities, but not between replicate population (transfer regime (ShC, StC and StB) nested with transfer experiment (one, three and six-day incubation) *P* = 0.001 and community replicate *P* = 0.2).

There were significant changes in biofilm and phenotype diversity indicated by the Simpson's reciprocal index (Figure 4.14, TK-HSD, α = 0.05). The six-day static community and static biofilm transfers show a significant decrease in biofilm and phenotypic species diversity compared to the initial soil-wash community (SW mean biofilm 1/D 9.26 ± 1.3 and

mean phenotype 1/D 5.43 ± 0.91; 6-D StC mean biofilm 1/D 3.59 ± 1.2 and mean phenotype 1/D 2.42 ± 0.8 1/D; 6-D StB mean biofilm 1/D 2.16 ± 0.9 and mean phenotype 1/D 2.42 ± 0.8). There was a significant decrease in diversity in all biofilm transfer experiments utilising the biofilm data, and a decrease in phenotypic diversity but this was not significant (1-D StB mean biofilm 1/D 4.63 ± 0.6 and mean phenotype 1/D 4.22 ± 1.9; 3-D StB mean biofilm 1/D 5.79 ± 1.1 and mean phenotype 1/D 3.48 ± 0.5). A similar pattern was found in the static community experiments, but these differences were not significant. There was also significant differences in diversity in the shaken community experiments, with the biggest decrease in biofilm diversity found in the one-day transfer experiment, and all shaken transfer experiments had significantly lower biofilm diversity than the initial soil-wash (1-D ShC mean biofilm 1/D 3.61 ± 1.1; 3-D ShC mean biofilm 1/D 5.43 ± 0.9 and 6-D ShC 1/D 6.21 ± 0.33). The same pattern was found in the phenotypic diversity in the shaken transfer communities, but differences were not significant. Results from the Shannon index displayed the same trend, but no differences were significant due to variation between replicate communities. Species indices confirmed changes in diversity between the initial soil-wash and final transfer communities, suggesting selection pressures were significant enough to effect community composition.

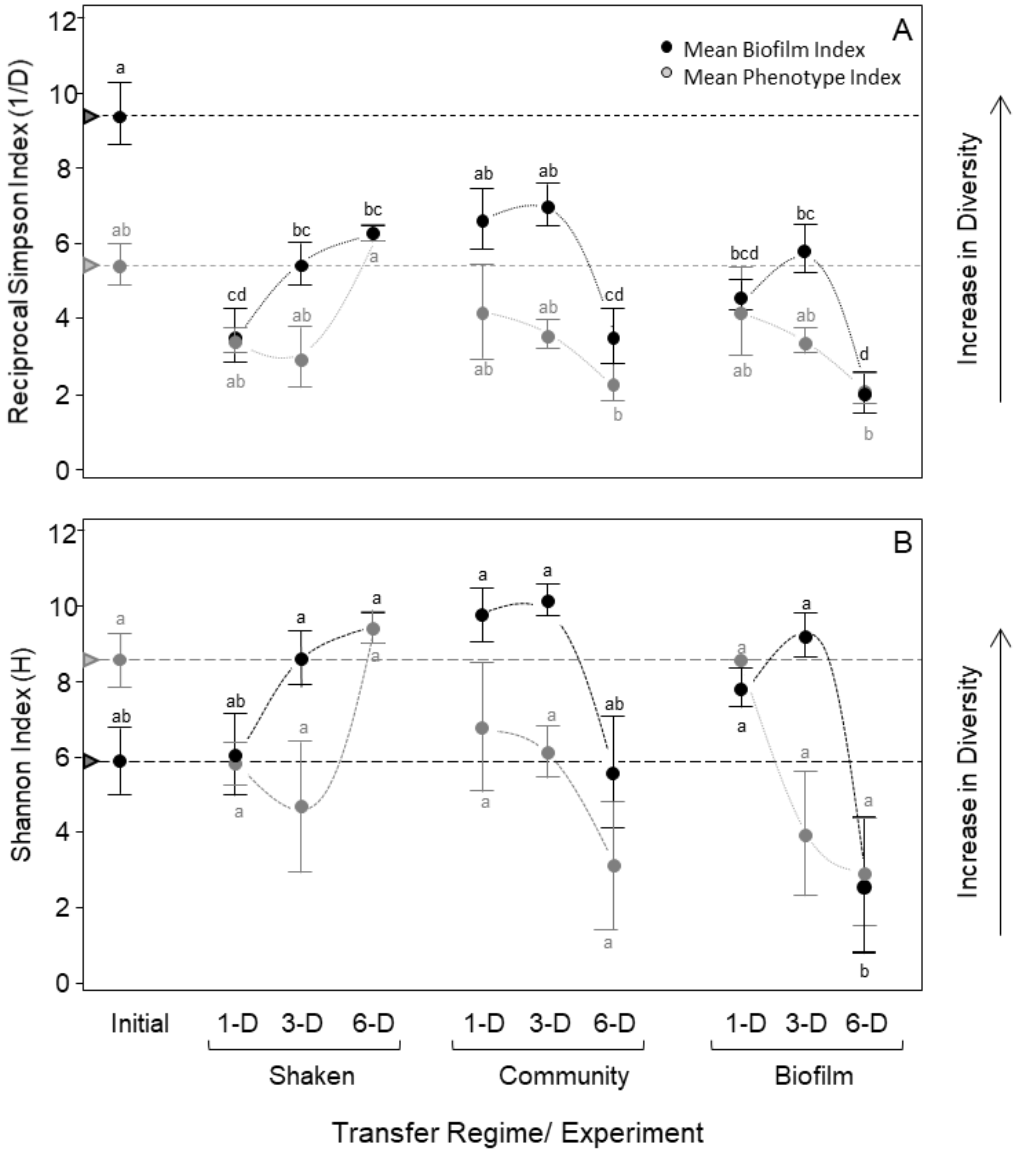


Figure 4.14. Simpson reciprocal and Shannon species indices comparing species diversity between communities. The Simpson reciprocal index (Panel A) and Shannon index (Panel B) were used to indicate changes in diversity between the initial soil-wash community and all final transfer communities. Indices were calculated using the mean biofilm (black circles) and mean phenotype (grey circles) data for each community. In both indices the higher the index value, the higher the diversity within the community. Replicate community means ± SE are shown, means not connected by the same letter a significantly differed. Trend lines are descriptive only. This data has been used in the Jerdan *et al*. (2020) publication.

The species indices calculated from the biofilm and phenotype data was used to further explore the relationship between diversity and productivity. Spearman's correlations found no significant correlation between community productivity (OD_{600}) and species diversity

across all treatments (ρ: community productivity and diversity determined by phenotype data P = 0.07; and between community productivity and diversity determine by combined biofilm data P = 0.33, nonetheless in particular six-day community and biofilm static treatments diversity was lower). This further indicates the complex relationship between community productivity and diversity within microbial communities.

4.2.5 Significant changes are found in productivity throughout the liquid column of the initial soil-wash and six-day transfer communities.

There were clear changes in community productivity, biofilm-associated and phenotypic traits across all serial-transfer experiments. The most significant changes were found in the six-day static community and static biofilm transfer communities. As a result, the six-day transfer communities were taken forward for further analysis. Visual observations and results from the combined biofilm assay suggested biofilm structure had significantly changed as a result of the serial transfer experiments. However, all communities retained a turbid liquid column, suggesting the low-O_2 liquid column was still supporting significant growth, even when the selection regime favoured biofilm-formation (biofilm only transfer). Biofilm research focusses on taxonomic make up and characteristics of the biofilm community, however in nature biofilms are commonly in contact with liquid where planktonic cells reside and may interact with the biofilm community. Productivity measurements in microcosms will be influenced by an increase in biofilm-formation and growth, however this does not distinguish the contribution of biofilm productivity and the productivity from the liquid column community. The liquid column community was further explored to determine how selection within O_2 limiting conditions impacts productivity below the biofilm. Many isolates capable of A-L interface biofilm-formation had turbid liquid columns suggesting isolates were cable of colonising both regions within microcosms, therefore the coalescing biofilm and liquid column community could be interacting and influence overall community productivity.

The cell localisation assay developed to investigate cell distribution throughout the liquid in Chapter 3 was used to investigate productivity throughout community microcosms. Differences in cell distribution between the six-day transfer communities and the initial soil-wash community were expected, with lower productivity found in the liquid column of the biofilm-transfer communities, where biofilm-formation is selected for. Many community isolates were capable of A-L interaction biofilm-formation and liquid column colonisation, suggesting isolates could migrate between the two coalescing communities. To investigate, isolates were first characterised for swimming motility, as migration between the liquid

column and A-L interface would require flagella-mediated motility (random diffusion is insufficient as demonstrated by the flagella deficient *P. fluorescens* SBW25 mutants in Chapter 3). Transplant experiments were developed to compare community productivity and biofilm strength when samples were transplanted from the biofilm or liquid column community and incubated in fresh microcosms. The presence of motile community members, and the ability for re-colonise both regions in fresh microcosms by both community samples would suggest migration between the biofilm and liquid column.

Final transfer communities became vertically stratified with static incubation, and cells colonised the A-L interface through biofilm-formation and occupied the lower liquid column with planktonic growth. All six-day transfer communities demonstrate a significantly higher relative cell density (relative OD_{600}) in the top 1ml of the liquid column where the biofilm community was present (Figure 4.15, TK-HSD, $\alpha = 0.05$). Both static six-day transfer communities (community and biofilm) demonstrated a greater level of cell enrichment to the top 1ml compared to the initial soil-wash and six-day shaken community. In the initial soil-wash cell density is higher in the top 1ml compared to the middle of the liquid column (2^{nd} – 5^{th} ml), however cell density was significantly higher in the bottom 1 ml of the liquid column, suggesting the presence of non-motile cells or obligate anaerobes. To further investigate, the productivity of the first 1 ml (biofilm community) and the pooled productivity of the liquid column (2^{nd} – 5^{th} ml) was compared between communities. There were significant differences in productivity between all communities in both the top 1ml sample and mid-column region, and in all communities the mid-column region had significantly higher productivity than the top sample containing the biofilm community (Figure 4.16, TK – HSD, $\alpha = 0.05$). The six-day biofilm transfer demonstrated the highest biofilm productivity (top 1ml) followed by the six-day static community, six-day shaken community and initial soil-wash respectively (6-D StB 1.72 ± 0.01; 6-D StC 1.53 ± 0.01; 6-D ShC 1.24 ± 0.02; SW 1.04 ± 0.01, relative OD_{600}, TK-HSD, $\alpha = 0.05$). The converse was found in the cumulative productivity of the liquid column (2^{nd} – 5^{th}) with the initial soil-wash community showing the highest mid-column productivity, and the six-day biofilm community having the lowest mid-column productivity (6-D StB 3.2 ± 0.04, 6-D StC 3.5 ± 0.02, 6-D ShC 3.6 ± 0.02 and SW 3.8 ± 0.01, relative OD_{600}, TK-HSD, $\alpha = 0.05$).

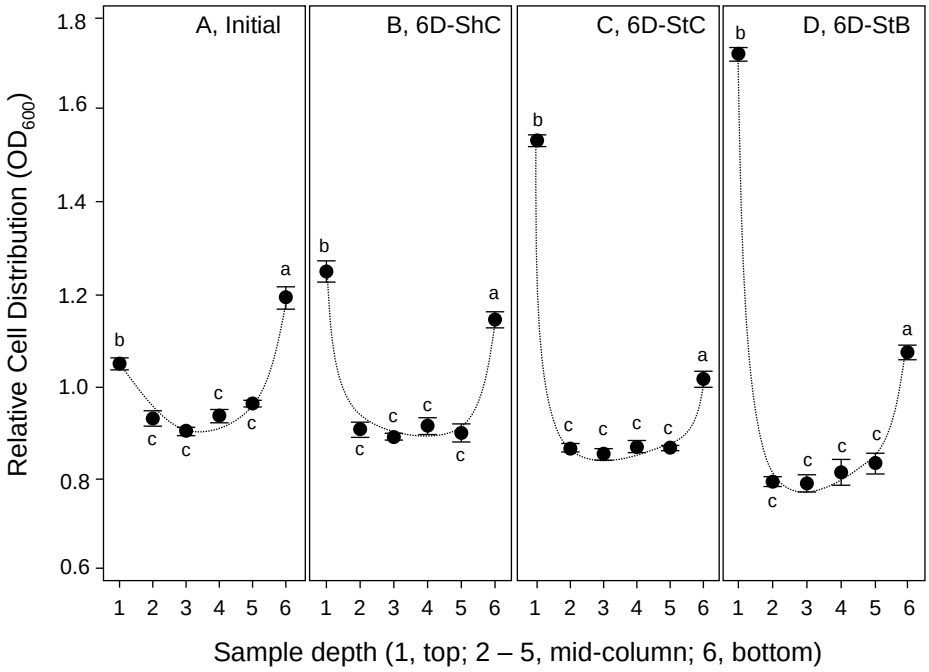


Figure 4.15. Cell distribution throughout the liquid column of initial soil-wash and six-day transfer communities. Cell distribution (OD_{600}) throughout the liquid column was explored by measuring cell density for every 1 ml sequentially down through the liquid column, where the first ml contains the biofilm community. The six-day transfer communities show significant cell enrichment to the top 1 ml compared to the lower liquid column (Panel B = six-day shaken community transfer, Panel C = six-day static community transfer, Panel D = six-day static biofilm transfer) compared to the initial soil-wash community (Panel A) which had significant cell enrichment at the bottom of the liquid column compared to the biofilm region. Means ± SE (n = 5) are shown, means not connected by the same letter are significantly different (TK-HSD, $\alpha = 0.05$). Trend lines are descriptive only. This data has been used in the Jerdan *et al*. (2020) publication.

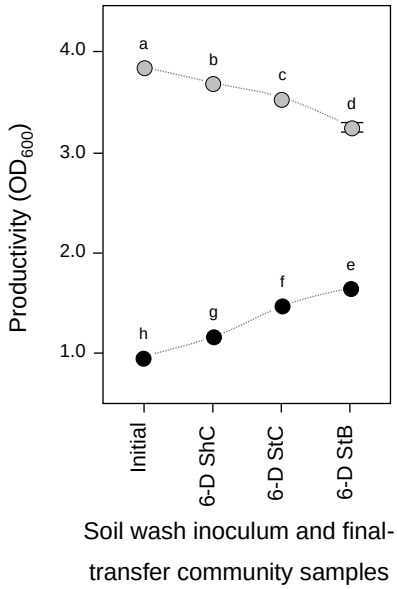


Figure 4.16. Comparison of community enrichment to the top of the liquid column and throughout the mid liquid column. Cell localisation (OD_{600}) to the top 1 ml of the liquid column (black circles) and the mid column region (grey circles, sum of 2^{nd} – 5^{th} ml) was compared between the initial soil-wash community and the six-day transfer communities after 24 hrs of static incubation. Means ± SE (n = 5) are shown, but for clarity error bars are not shown where they are smaller than the mean symbol. Means not connected by the same letters are significantly different (TK-HSD, α = 0.05). Trend lines are descriptive only. This data has been used in the Jerdan *et al*. (2020) publication.

Further exploration of cell distribution was carried out with individual isolates from the initial soil-wash and six-day communities. Community isolates were predicted to show differences in cell distribution to reflect difference in cell distribution at the community level, with the initial soil-wash expected to contain isolates with significantly lower cell localisation to the high-O_2 region. Cell localisation assays were carried out with isolates (n = 8) from each community, and isolates showing the highest and lowest cell localisation were taken forward for analysis (n = 4). There were significant differences between cell localisation to the top 1 ml between isolates from both the soil-wash and six-day transfer communities after 24 h of static incubation (Figure 4.17, TK-HSD, α = 0.05). Initial soil-wash isolates demonstrated cell enrichment below 1.5 OD_{600}, while isolates showing the highest cell enrichment in the six-day transfer communities show higher cell density, between ~2 and 2.5 OD_{600}. All six-day communities contained isolates showing significantly higher cell enrichment compared to the lowest enriching isolates, even in the shaking transfer experiment where no O_2 gradient is established. The six-day biofilm transfer which samples from the biofilm only retained

isolates with no significant cell localisation to the top of the liquid column, where cell distribution was even throughout the entire liquid column. Enrichment at the top and mid-column distributions were negatively correlated for six-day static biofilm isolates (n = 8 tested, ρ = -0.88, *P* = 0.01) but not for the six-day shaken and static community, or the soil-wash isolates (*P* = 0.18, 0.53, 0.82). Cell distributions of all eight isolates can be found in appendix A1.3.

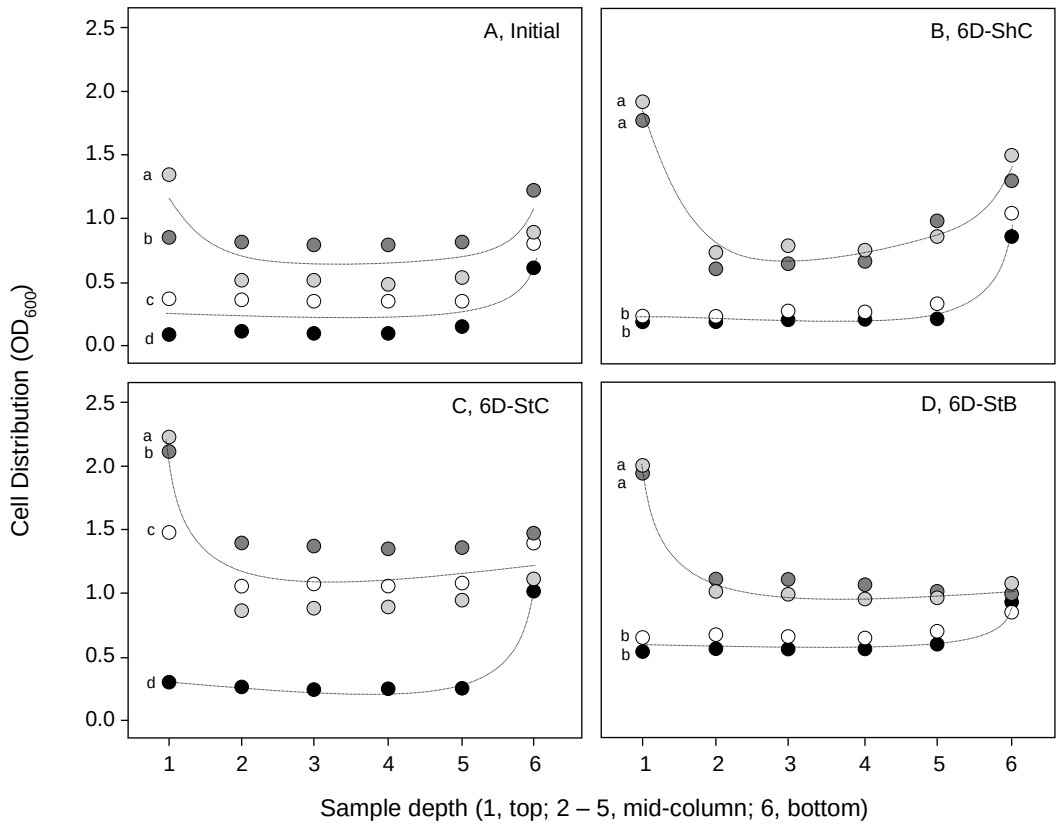


Figure 4.17. Cell distribution throughout the liquid column of individual isolate from the initial soil-wash and six-day transfer communities. Cell distribution throughout the liquid column was explored in individual isolates from the initial soil-wash and six-day transfer communities (Panel A = initial soil-wash, Panel B = six-day shaken community transfer, Panel C = six-day static community transfer, Panel D = six-day static biofilm transfer). Cell density (OD_{600}) was measured for every 1 ml sequentially down through the liquid column, where the first ml contains the biofilm community (representative strains, *n* = 4, are indicated by black, dark and light grey, and white circles). Means ± SE (n = 5) are shown, but for clarity error bars are not shown where they are smaller than the mean symbol. The mean of enrichment (top 1 ml) were compared and means not connected by the same letters are significantly different (TK-HSD, α = 0.05). Trend lines are descriptive only. Cell distribution for all 8 isolates tested from each community can be found in Appendix A1.3 This data has been used in the Jerdan *et al*. (2020) publication.

As selected communities and many individual isolates were capable of biofilm-formation and colonising both the lower liquid column, it was hypothesised cells were capable of migration between the two regions. For cells to access the high-O_2 region where biofilm-formation occurs, swimming motility is required. Isolates form the initial soil-wash and six-day transfer communities were tested in plate-based swimming motility assays (see Appendix A1.4 for motility data). Here, 28 of the 32 isolates tested (n = 8 from each community) were found to be motile, suggesting cells can both access and migrate between the liquid column and high-O_2 region. The non-motile isolates were found in the initial soil-wash and six-day shaken community.

Transplant experiments were developed where samples from the biofilm community and the lower liquid community were transplanted to fresh static microcosm. After three days productivity (OD_{600}) and biofilm strength was measured. If both regions were re-colonised in fresh microcosms this would suggest community members were capable of migration between the two regions. When both the biofilm and liquid column community was re-incubated in static liquid microcosms, both regions were re-colonised. There was no significant differences in total microcosm productivity between the fresh microcosms, indicating source of transplant sample (liquid column or the biofilm community) was not significant (Figure 4.18, T-HSD, $\alpha = 0.05$), except from the six-day shaken transfer community where the productivity of the biofilm community produced higher productivity in fresh microcosms compared to the liquid column (biofilm community 1.97 ± 0.01 and liquid column 1.87 ± 0.01, $OD_{600,}$ TK-HSD, $\alpha = 0.05$). There was a significant difference in biofilm strength (grams) in the six-day shaken and six-day static biofilm communities, with the biofilm sample having significantly higher strength compared to the liquid column community (6-D ShC biofilm community 0.37 ± 0.02 and liquid column community 0.05 ± 0.02; 6-D StB biofilm community 0.32 ± 0.01 and liquid column community 0.12 ± 0.01, grams, TK-HSD, $\alpha = 0.05$). These results suggest that community members are capable of migrating between the biofilm and liquid column community, however some isolates situated with the biofilm community produce stronger biofilms than isolates found within the liquid column.

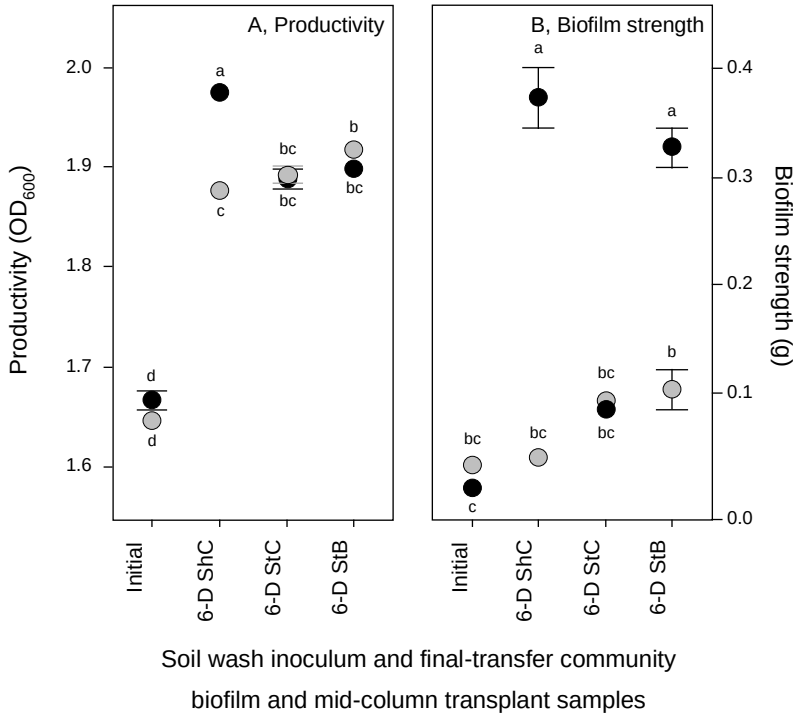


Figure 4.18. Community samples taken from the liquid column of biofilm can re-colonise both regions in fresh static microcosms. Transplant experiments were used to determine if isolates were capable of migrating between the liquid column and biofilm community, and therefore suggesting the two coalescing communities were interacting. Sample from the biofilm (black circles) and liquid column (grey circles) were taken from the initial soil wash and six-day transfer communities and re-inoculated into fresh microcosms, and community productivity (OD_{600}, Panel A) and biofilm strength (g, Panel B) were measured after three days. Means ± SE (n = 5) are shown, but for clarity error bars are not shown where they are smaller than the mean symbol. Means not connected by the same letters are significantly different (TK-HSD, α = 0.05). This data has been used in the Jerdan *et al*. (2020) publication.

Relative productivity in all community microcosms was dominated by the liquid column, despite the low-O_2 conditions. To confirm the effect of oxygenation on community productivity, the initial soil-wash and six-day transfer communities were subjected to three oxygenation conditions and community productivity (OD_{600}) was compared. Communities were incubated under high-O_2 conditions with shaking incubation, normal static oxygenation conditions where an O_2 gradient is formed, and very-low-O_2 conditions in anaerobic canasters. In all community samples, productivity was significantly lower in very-low-O_2 conditions (Figure 4.19, TK-HSD, α = 0.05) and there were no significant differences in productivity in very-low-O_2 conditions between the community samples (Wilcoxon, $P > 0.05$).

Communities show the highest productivity in high-O_2 conditions, with a small decrease in productivity in static conditions where and O_2 gradient is established.

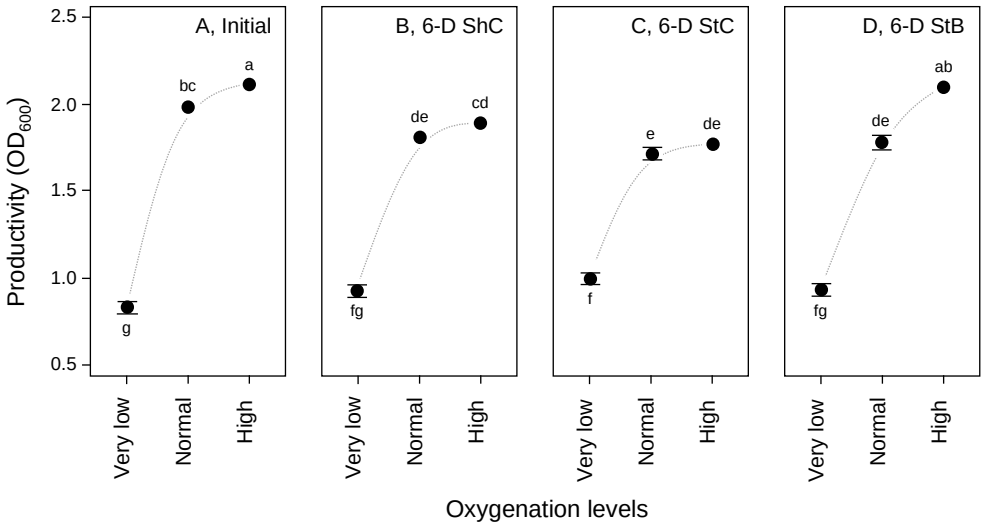


Figure 4.19. High-O_2 conditions results in increase community productivity. Productivity (OD_{600}) of the initial soil-wash (Panel A) and six-day transfer communities (Panel B = six-day shaken community transfer, Panel C = six-day static community transfer, Panel D = six-day static biofilm transfer) were compared under very-low, normal and high O_2 conditions. Productivity was measured after three days incubation under shaking conditions to provided high O_2 levels throughout the liquid, static condition to provide normal O_2 condition where an O_2 gradient is established, and static incubation in anaerobic canisters to provide very low O_2 conditions. Means ± SE (n = 3) are shown, but for clarity error bars are not shown where they are smaller than the mean symbol. Means not connected by the same letters are significantly different (TK-HSD, α = 0.05). This data has been used in the Jerdan *et al*. (2020) publication.

Individual community isolates were also compared under different oxygenation conditions. There were significant increases in individual isolate productivity in high-O_2 conditions, but no differences between strains under normal or very-low O_2 conditions (Kruskal-Wallis, high-O_2, $P = 0.01$; very-low O_2 and normal conditions, $P > 0.05$). Both community and individual isolate response data was combined and productivity under high-O_2 conditions was compared to productivity under normal conditions, and normal conditions with very-low O_2 conditions (Figure 4.20). A small number of individual isolates demonstrated a small increase in productivity under normal conditions compared to high-O_2 conditions, but no individual isolates or communities had higher productivity in very-low-O_2 conditions compared to normal conditions. These results confirm community productivity is optimal in highly oxygenated conditions and significantly lower in low-O_2 conditions, suggesting isolates

colonising the liquid column in community static liquid microcosms are subjected to slower growth rates than occupying the high-O_2 region through biofilm-formation.

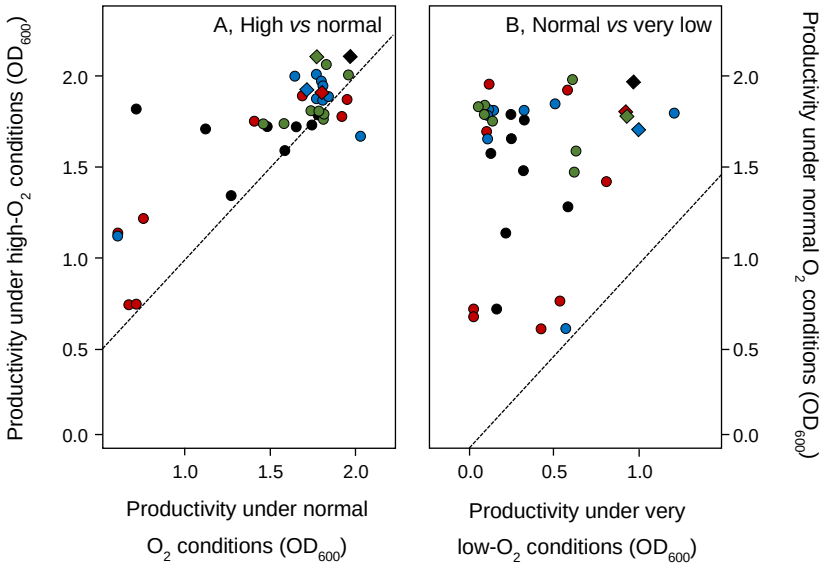


Figure 4.20. Productivity of communities and individual community isolates are affected by oxygenation. The productivity of the initial soil-wash (black), six-day shaken (red), six-day static community (blue) and six-day static biofilm transfer community (green) were incubated in high, normal and very-low O_2 conditions. The productivity of individual isolates (circles) and the community samples (diamonds) were combined and pairwise comparison conducted between high-O_2 and normal conditions (Panel A), and normal and very-low O_2 conditions (Panel B). The dotted line in each panel indicates where productivity is equal under both conditions. The mean ± SE (n = 3) is shown, but for clarity error bars smaller than the symbol size are not shown. This data has been used in the Jerdan *et al*. (2020) publication.

4.2.6 Initial microscopy and strain identification suggest changes in biofilm matrix, structure and species composition of initial soil-wash and six-day static transfer communities

Results from the combined biofilm assay and visual observations indicate biofilm structure of communities undergone serial-transfer in static conditions have significantly changed. This suggests composition of the biofilm including extracellular matrix substances (EPS) have also altered. Although exploring the EPS and taxonomic make-up of the community samples

were not in the original research aims, an opportunity arose on an Erasmus + traineeship for confocal scanning laser microscopy, scanning electron microscopy and MALDI-TOF MS analysis. This analysis was time and sample dependent, however provides an initial indication of changes in biofilm structure and EPS, and species identification.

Images taken of the initial soil-wash and three six-day transfer communities (1 population selected from each of the replicate communities undergone selection) provide visual evidence of changes between communities (Figure 4.21). Samples incubated overnight with shaking to provide inoculum for ongoing experiments demonstrate the static community and static biofilm transfer can form A-L interface biofilms within a short time period under shaking conditions. No A-L interface biofilm-formation occurred in overnight cultures of the initial soil-wash or the six-day shaken communities, with only a small ring of attached cells around the glass vile above the meniscus. Images taken of three-day biofilm experiments also demonstrated differences between communities. The initial soil-wash community produced a very thin and unstructured looking A-L interface biofilm. The six-day shaken community did produce a more structured looking biofilm, but still appeared thin. The six-day static community and biofilm transfers produce physically cohesive, elastic A-L interface biofilms which appeared much thicker. The liquid column of these communities also had visual differences in colour, and the initial soil-wash community appeared to have a more turbid liquid column

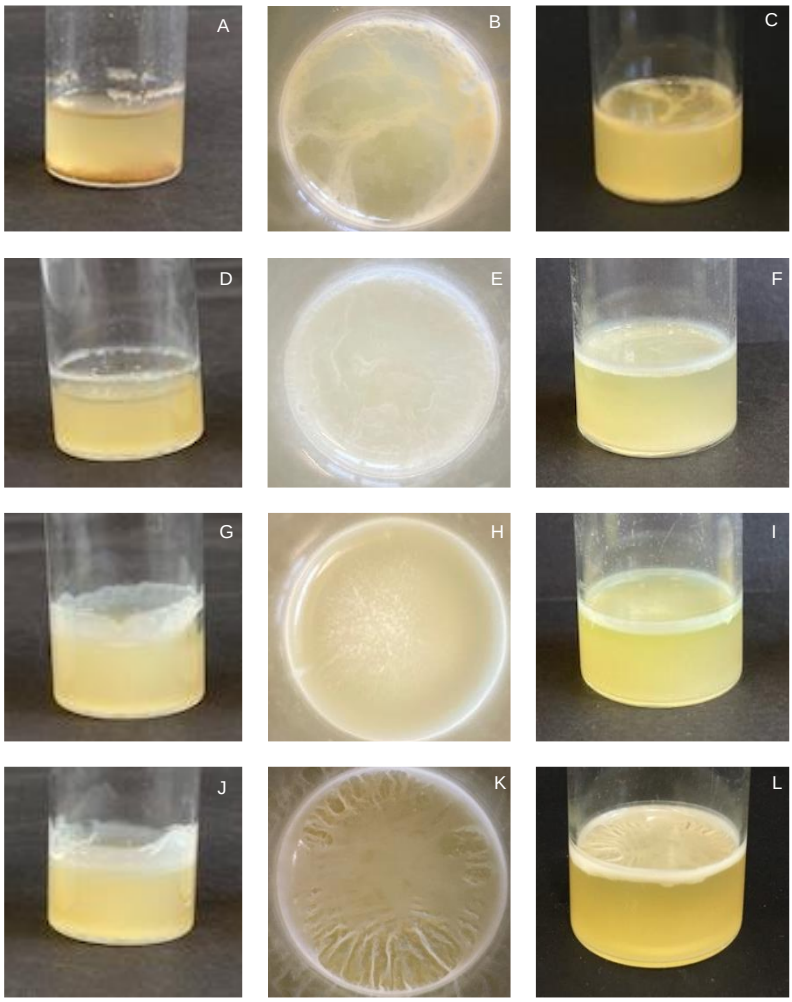


Figure 4.21. Image comparison of the initial soil-wash biofilms and six-day transfer communities. Images were taken of the initial soil wash (A – C), six-day shaken community (D – E), six-day static community (G – I) and six-day static biofilm community (J – L) after overnight shaking incubation (left images) and after three days of static incubation showing the surface of the biofilm (middle images) and a side on view of the biofilm (right images). These images are descriptive only.

To further investigate changes in the structural differences of the six-day static transfer communities and the initial soil-wash, confocal scanning laser microscopy (CSLM) and scanning electron microscopy (SEM) was performed at the National Academy of Sciences, Ukraine. This analysis was limited to the duration of the two weeks Erasmus + traineeship, and only the initial soil-wash, and the two static six-day transfer communities were available to analyse. Combinations of EPS and cell stains were applied for CLSM analysis (Figure 4.22, A and B). CLSM 3D images suggests the EPS matrix of the initial soil-wash community was dominated with red signal, suggesting extracellular DNA (eDNA). The six-day static

biofilm and six-day static community was dominated with a blue signal, EPS stained with calcofluor suggesting a cellulose like polymer. This six-day biofilm community also appeared more densely packed with cells (green signal) and EPS compared to the initial soil-wash. This assessment was made by eye only, and signal quantification is needed to confirm results. This suggests changes in biofilm structure and EPS composition between the initial soil-wash and six-day static transfer communities, however insufficient replicate analysis was performed to conclude these changes. Similarly, initial SEM analysis show a cross section of the six-day biofilm transfer community in which there are visible layers and structure within the biofilm. The initial soil-wash easily fragmented upon sampling, was much thinner, and little structure and layering was observed. Similar to the CLSM analysis, further replicate analysis is needed to confirm these structural differences with SEM.

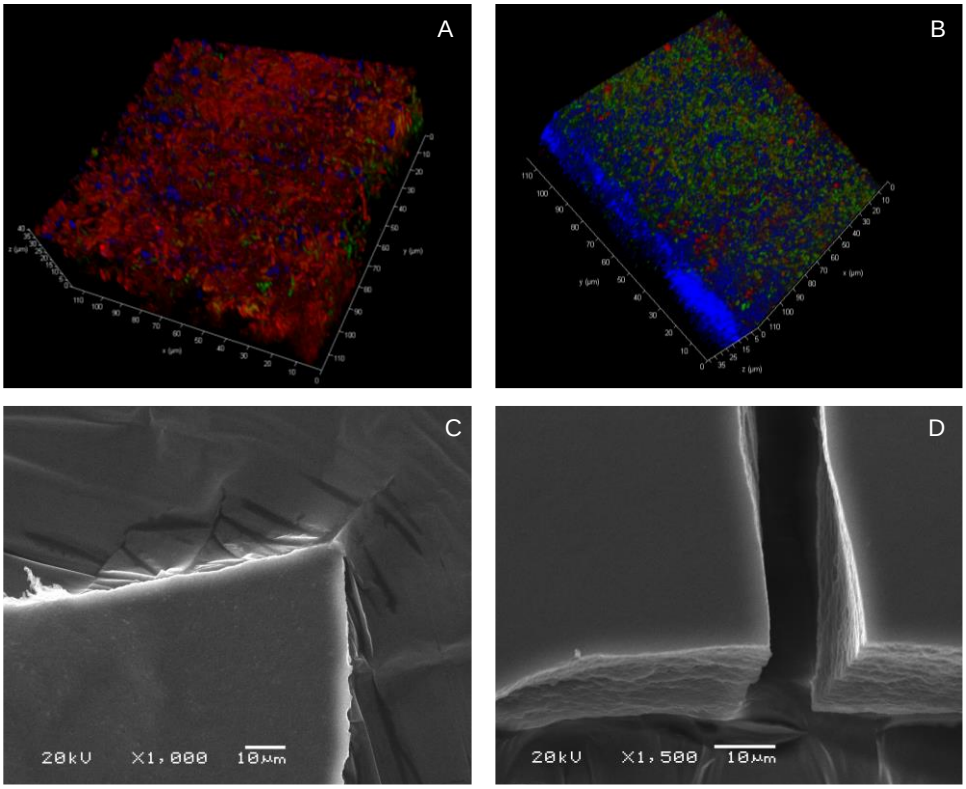


Figure 4.22. Confocal and scanning electron images comparing biofilms produced by the initial soil-wash and six-day static biofilm community. Descriptive images of the initial soil-wash (left-hand images) and 6 – day biofilm transfer community (right-hand image) were taken using confocal scanning laser microscopy (top images) and scanning electron microscopy (bottom images). For confocal images biofilm sample were first stained, and within these images green signal are stained cells, red signal is extracellular DNA and blue signal is cellulose-like polymers.

Isolates (n = 4) from the initial soil-wash and six-day static community and static biofilm community were analysed using MALDI-TOF mass spectrometry. Of the 12 isolates analysed, 11 were identified at species level based on the bioMérieux species database. Four isolates from the initial soil-wash were identified as *Leclereia adecarboylata, Lelliottia amnigena, Pentoea agglomerans* and *Serratia plymuthica.* From the six-day biofilm transfer community, three isolates were identified as *Serratia proteamaculans* and the fourth as *Pseudomonas veronii*. Of the four isolates tested from the six-day static community, only three could be identified, all as *Pseudomonas veronii.* MALDI-TOF MS identification is made based on ribosomal proteins, therefore where there is no match in the database no identification is made. However, it is not clear whether multiple species in a complex genus like pseudomonads may have the same or similar ribosomal proteins. Further species identification would be needed to assess true changes in species diversity, but with the species identified and similar colony morphologies between isolates in final transfer communities, diversity has likely changed.

4.2.7 Communities undergone selection within a O_2 heterogenous environment are more resistant to invading species

Species diversity and community characteristics play an important role in community resistance and resilience, in particular community invasibility. Some microcosm based experiments support the insurance hypothesis (Yachi and Loreau, 1999) concluding a more diverse community is likely to contain taxa with an appropriate trait allowing for increased community resilience, resistance and stability(Jaing and Morin, 2004; Eisenhauer, Scheu and Josset, 2012). However, other studies suggest susceptibility to invading species is linked to community interactions, where increased species interaction create resistance to invading species (BurmØlle *et al*., 2006) or the phylogenetic distance between the community and invader (Li *et al.,* 2019). The insurance hypothesis would suggest that communities undergone selection resulting in lower diversity would be more susceptible to invasion. This would suggest the initial soil-wash was a more resistant community with more taxon. However, transfer communities show adaption to the microcosm environment, and contain competitive phenotypes. To test if trait-based diversity or adaption and interactions are important in community resistance of A-L interface biofilm communities, communities were be subjected to invading species. *P. fluorescens* SBW25 wild-type and Wrinkly spreader mutant (WS) were neutrally marked with the *lac*Z reporter marker and represented invading strains in the initial soil-wash community and six-day transfer communities. If the

insurance hypothesis was followed, the six- day shaken community and initial soil-wash would be more resistant of invasion by *P. fluorescens* SBW25 as these communities contain higher species diversity. However, within the six-day static community and static biofilm communities contain a higher percentage of biofilm forming members with increased biofilm characteristics and demonstrate more competitive interactions, suggesting the WS mutant would have more isolates to compete against for access to the high-O_2 region.

To undertake pairwise fitness assays with community samples and invading strains, *lac*Z marked wild-type SBW25 and WS mutants were constructed. Here, successfully marked strains will appear blue on plates containing X-gal and can be distinguished from the community. The *lac*Z marked wild-type SBW25 strain from Zhang and Rainey (2007), was utilised to construct a WS-*lac*Z, where SBW25-*lac*Z was electroporated with the WS wspF allele. Once constructed, the *lac*Z strains were tested in pairwise fitness assays with the isogenic strain to test for neutrality (Figure 4.23, TK-HSD, α = 0.05). WS-*lac*Z mutants were observably smaller than archetypal WS mutant colonies, and there was a small significant increase in the competitive fitness (W) of the WS-*lac*Z (WS-*lac*Z W - 1.28 ± 0.04). As reported in Zhang and Rainey (2007) SBW25-*lac*Z was neutral, with no significant increase in competitive fitness over wild-type SBW25 (wild-type-lacZ W 0.94 ± 0.04). However, the small increase in fitness of the WS-*lac*Z is not a concern and provides a better competitive strain to test within the communities.

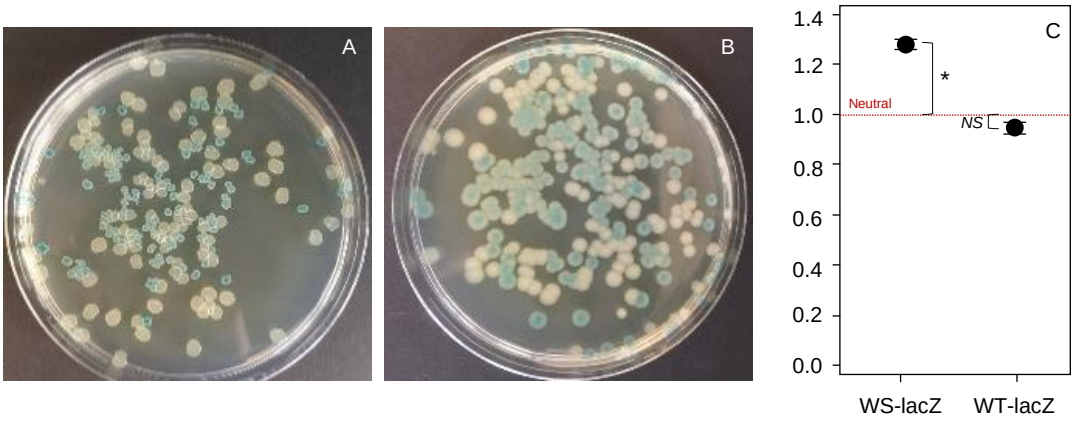


Figure 4.23. *P. fluorescens* SBW25 wild-type and WS mutant *lac*Z marked strains. SBW25-*lac*Z (Zhang and Rainey, 2007) was used to construct WS-*lac*Z mutant, and both appear blue on X-gal plates and can be distinguished from the archetypal strain (A = WS-*lac*Z, B = SBW25-*lac*Z). Both marked strains were tested in pairwise competitive fitness assays with the archetypal strain to test for neutrality. A significant increase above one (red line) suggests an increase in competitive fitness of the marked strains. Means ± SE are shown, asterisks indicate a significant increase in fitness above one (T-test, $P < 0.05$).

Pairwise competitive fitness assay were carried out with an initial ratio of 1:1 (or as close to 1:1 as possible) with the community samples and the invading strain, and incubated for three days with both static and shaking conditions (Figure 4.25, TK-HSD, α = 0.05). The WS mutant is known to have a competitive advantage over non-biofilm forming competitors in static conditions, however this advantage is lost in shaking conditions where biofilm-formation is not favoured, and the wild-type SBW25 strain performs better. The fitness of the invading species was calculating using Malthusian parameters, with a fitness (W) significantly higher than one showing a competitive fitness and successful invasion of the community. The WS-*lac*Z mutant had a significant competitive fitness of above one when invading the six-day shaking community in static conditions (T-test, $P = 0.02$). SBW25-*lac*Z had no significant fitness advantage over the six-day shaken community in static conditions (T-test, $P = 0.07$), and suggests neither the community or invading strain had a competitive advantage. This was also found in the initial soil-wash community in static conditions when WS-*lac*Z was introduced to the community. In all other competitive fitness assays, the community samples had a competitive fitness over the invading species ($P < 0.05$), suggesting resistance against invasion from wild-type SBW25 and the WS mutant. The fitness of both invading strains under static conditions was significantly lower in the six-day static transfer and static biofilm communities (W = 0.26 - 0.33) compared to the initial soil-wash and six-day shaken community (W = 0.73 - 1.09), where the invading strain was almost completely inhibited in the static transfer communities. A similar observation was found in shaken conditions, with the SBW25-*lac*Z and WS-*lac*Z mutant having the lowest fitness in the six-day biofilm transfer community (TK-HSD, α = 0.05). These results suggest the six-day static community and static biofilm transfers were the most resistant to invasion despite showing the lowest trait-based diversity.

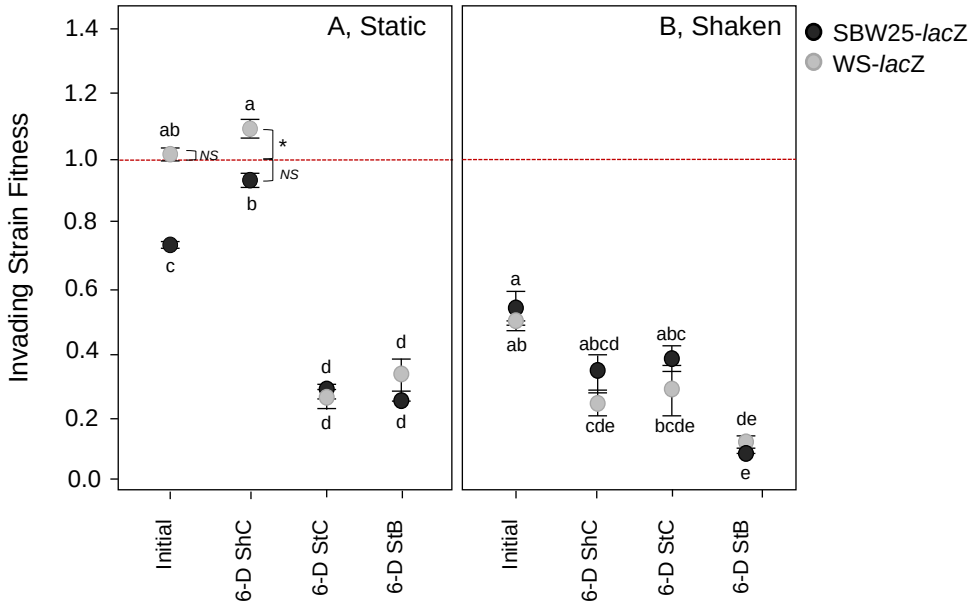


Figure 4.24 Six-day static transfer communities have increased resistance to community invasion compared to the initial soil-wash and six-day shaken community. Pair-wise fitness assays were carried out with the initial soil-wash or six-day transfer communities and *P. fluorescens SBW25* strains marked with the *lac*Z reporter marker which represented invading species. Replicate communities (n = 5) were incubated statically (Panel A) or with shaking (Panel B) with SBW25-*lac*Z (black circles) or WS-*lac*Z (grey circles) and plated after three days. The fitness of the invading strain is shown in each community sample. The red dotted line at 1 indicates a fitness of 1, where the fitness of each strain would be equal. A fitness of W<1 indicates that the strain is at a disadvantage. A fitness of W> 1 indicates the strain has the competitive advantage. Means ± SE are shown (n = 5), means not connected by the same letter within the same panel and statistically different (T-K HSD, α = 0.05). The significance of means close to one were tested, and means significantly higher than one are indicated with an asterisk (T-test, $P < 0.05$) Trend lines (dashed curves) are descriptive only.

Very few SBW25-*lac*Z and WS-*lac*Z colonies were found in the six-day static community and static biofilm pairwise fitness experiments, suggesting an inhibiting effect. To investigate further, spot-on-lawn interaction assays were conducted, with invading strains plated as lawns and community isolates (n = 8 from each community) spotted on top. More antagonistic and competitive interactions against the invading strains were found in the six-day static community and six-day biofilm transfer community compared to the initial soil-wash and six-day shaking community (Figure 4.25). Results from both invading strains were analysis, and a test of independence indicates that strain origin (ShC, StC, StB or intial) and interaction type (antagonistic, competitive and neutral) were dependent (*ChiSquared*, $P < 0.001$). Odds ratios were calculated against the initial soil-wash results, and it was

significantly more likely to find negative interactions (antagonistic or competitive) against the invading strain than no interaction in the six-day static community and six-day biofilm transfer community compared to the initial soil-wash, and the six-day shaking community had the same number of negative interaction as the initial-soil wash (6-D StB Odds ratio 35.2, P = 0.001; 6-D StC Odds ratio 15.4, P = 0.001 and 6-D ShC Odds ratio 1, P = 0.5). This result confirms that communities in which the SBW25-*lac*Z and WS-*lac*Z performed the poorest had the highest level of antimicrobial activity, possibly competitive and antagonist interactions.

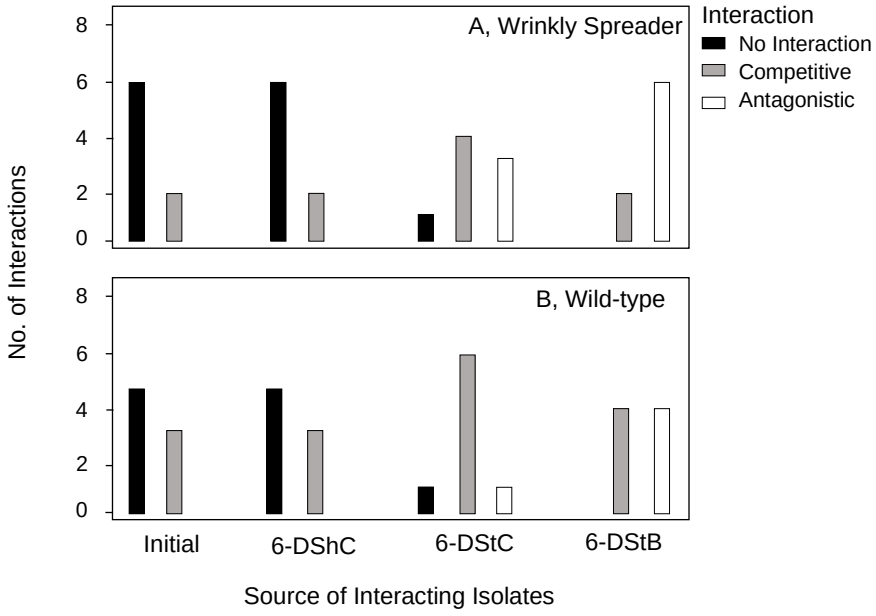


Figure 4.25. Increased competitive and antagonistic interactions increase resistance against invading species. Spot-on-lawn interactions assays were carried out with the invading strains (SBW25-*lac*Z and WS-*lac*Z) plated as a lawn, and community isolates (n = 8) from the initial soil-wash and six-day transfer communities spotted on top. Interaction results were recorded after 48 hrs, and data is shown as number of interactions (none = black bars, competitive = grey bars, antagonist = white bars) against WS-*lac*Z (Panel A) and SBW25-*lac*Z (Panel B). Chi-square test of independence indicates that strain origin (ShC, StC, StB or initial) and interaction type (antagonistic, competitive and neutral) were dependent (*ChiSquared*, P < 0.001).

4.3. Discussion

O_2 gradients are ubiquitous in nature, and single-species model systems demonstrate adaption to O_2 limiting conditions. Complex communities contain diverse species with different phenotypic characteristics, so understanding microbial community response to developing O_2 gradients is complex. Within this chapter the microcosms model system utilised to study adaptive radiation in *P. fluorescens* SBW25 and air-liquid interface biofilm-formation was successfully developed to study successional changes in multi-species A-L interface biofilm-forming communities. An appropriate soil model community was established, and conditions within static liquid microcosms provided a system to study the effects of O_2 gradients on the development and selection within community biofilms. A serial-transfer approach was used, and several transfer regimes were designed in which some favoured biofilm-formation more than others. Community changes were captured measuring community productivity (OD_{600}) and biofilm-associated traits. This was repeated at the individual-isolate-level, where isolates were compared utilising a series of phenotypic and behavioural assays to further investigate selective changes. The involvement of species interactions was also explored, as interactions are known to shape community function and emergent properties.

Through the short-term evolutionary experiments the low-O_2 liquid column continued to support growth, even when conditions favoured biofilm-formation. The influence of the liquid column on overall community productivity was explored, and transplant and motility experiments investigated the possibility of cell migration between the biofilm and liquid column, suggesting interactions and movement between the two coalescing communities. Finally, invasability of final transfer communities were compared with the initial soil-wash to investigate changes in community resistance as a result of selection in varying environmental conditions.

4.3.1 A microcosm model system for investigating heterogeneous O_2 limiting conditions and time in air-liquid interface biofilm communities

To investigate the effects of developing O_2 gradients and time limitations on community productivity and aggregated traits associated with air-liquid interface biofilm-formation an appropriate model system and model community was required. The microcosm model system was chosen, where upon static incubation of bacteria, environmental heterogeneity is developed through O_2 depletion (Koza *et al*., 2011). The O_2 gradient established creates a high-O_2 region directly below the A-L interface. This presents ecological opportunity and

strains capable of A-L interface biofilm-formation or flagella mediated aerotaxis can access the high-O_2 region for increased O_2 access (Koza *et al*., 2017). With shaking incubation O_2 is constantly introduced into the system, and the liquid column remains homogeneous (Madsen *et al.*, 2015). A soil-wash multi-species community was chosen as a model community for this research and is commonly used in biofilm and eco-evolutionary studies (Burmølle *et al*, 2006; Burmølle *et al*., 2007 Ren *et al*., 2015; Johansen *et al*, 2019). Soil microbial communities are likely to be organised in biofilms around plant roots, litter and soil particles (Burmølle, Hansen and Sørensen, 2007) so are relevant for biofilm studies. In addition, soil microbial communities are likely rich in pseudomonads, capable of A-L interface biofilm-formation (Ude *et al*., 2006). Initial experiments were performed with an aim of confirming the model community and system chosen is appropriate for studying changes in community A-L interface biofilms in O_2 limiting conditions. This required evidencing the establishment an O_2 gradient and A-L interface biofilm-formation in static conditions with the soil-wash community, and changes in community productivity in response to altered incubation conditions demonstrating a selecting effect.

After preparation of the soil-wash, samples were directly inoculated into liquid microcosms. This is an ecological relevant approach producing a semi-natural community (Blasche *et al*., 2017), where there is an assumption of prior coexistence and interactions between isolates, relevant for successional studies (Røder, Sørensen and Burmølle, 2016).Using the redox indicator methylene blue, the soil-wash community formed an O_2 gradient in static liquid microcosms, confirmed over a 24h period (Figure 4.2). This result is in agreement with the O_2 profiling within the *P. fluorescens* SBW25 system, where after 5 h a steep O_2 gradient established, with 0.1% of the normal dissolved O_2 below 1 mm (Koza *et al*., 2011). As O_2 profiling has been extensively researched using *P. fluorescens* SBW25 by different research groups (Koza et al., 2011; Loudon *et al*., 2016), the prompt reduction of methylene blue was sufficient to satisfying evidence of an O_2 gradient in community microcosms. Static incubation for 24 h also confirmed the formation of A-L interface biofilms in soil microcosms, with planktonic growth remaining in the liquid column. Many other studies utilising soil microbial communities confirm the formation of L-S biofilms in microtiter plates (Burmølle *et al*, 2006; Burmølle *et al*., 2007 Ren *et al*., 2015), but this system confirms that soil microbial communities are also an appropriate model communities for studying A-L interface biofilms.

Soil-wash samples were plated on different media types for a visual assessment of colony diversity. KB* nutrients produced the highest CFU and most visual colony diversity (Figure 4.1). It is estimated that only 1% of soil microbial isolates are culturable in laboratory

conditions (Amman, Ludwig and Schleifer, 1995) with research needed to develop suitable nutrients to discover currently un-culturable soil microbes. It was therefore expected that soil-wash inoculum provides lower-diversity than natural occurring soil aggregated samples (Howard *et al*. 2017). Storage of the starting inoculum was also an important experimental design, to provide experiments with the same, or as close to, starting community. There is evidence of evolution within strains, loss of genetic material and even loss in biofilm lifestyle determinants of bacteria in laboratory environments (Eydallin *et al.,* 2014). Colony counts and visual comparison of colony morphology diversity was made between the initial soil sample and after short and prolonged storage at -80˚C, and no significant changes were observed. Isolates selected from the initial soil-wash were taken from soil-wash samples stored at -80˚C for 14 days to account for any loss of diversity through the freezing and incubation process. To further confirm no significant differences in species diversity due to storage at -80˚C, and to capture true starting diversity of the soil-wash community 16s rRNA sequencing and metagenomics is needed for species identification. Similarly, microscopy could be used to determine differences in cell morphology to further understanding diversity changes Plates were also checked for the presence of protists, bacteriophage, nematodes and fungi, however further experimentation would be needed to confirm any presence through microscopy or filtering and spreading bacterial lawns to allow identification of phages. Soil microbial diversity is still poorly understood however bacteriophage and protists are known to drive microbial diversity (Fierer, 2017), and are known to drive ecological and evolutionary processes in microcosm laboratory experiments (Koskella and Brockhurst, 2014). The lack of protists or bacteriophage within this soil-wash suggests they did not survive inoculum preparation and storage, and KB media is designed to support bacteria growth, in particular strains of fluorescent pseudomonads (King, Ward and Raney, 1954).

Serial-transfer experiments performed with soil microbial communities, measuring changes in taxanomic diversity with 16s ribosomal RNA have shown that soil communities cultured in laboratory conditions show very high levels of diversity (Goldford *et al*., 2018). Across replicate samples 16S sequencing revealed community composition covering ~23 groups at the taxonomic level, with Enterobacteriales, Pseudomonadales, Flavobacteriales and Burkholderiales dominating composition, and between 100 – 1290 unique exact sequence variants (ESV) within soil and plant microbial communities. MALDI-TOF mass spectrometry identified 11 of 12 isolates from the soil communities within this research, also revealing a diverse range of bacteria species. The 11 identified species were all proteobacteria, from two different orders, four different families and five different species (Figure 4.26). There are also prevalent colonies of suspected *Bacillus* species, such as *Bacillus mycoides.* This is a

limited insight to the true diversity of the soil-wash community, where I suspect many other species identified with further analysis. From isolates identified both aerobic (*P. veronii*) and facultative anaerobes (*Serratia* spp) are present suggesting competition for the high-O_2 region in static liquid microcosms is high, but some strains cable of maintaining growth in the low-O_2 liquid column.

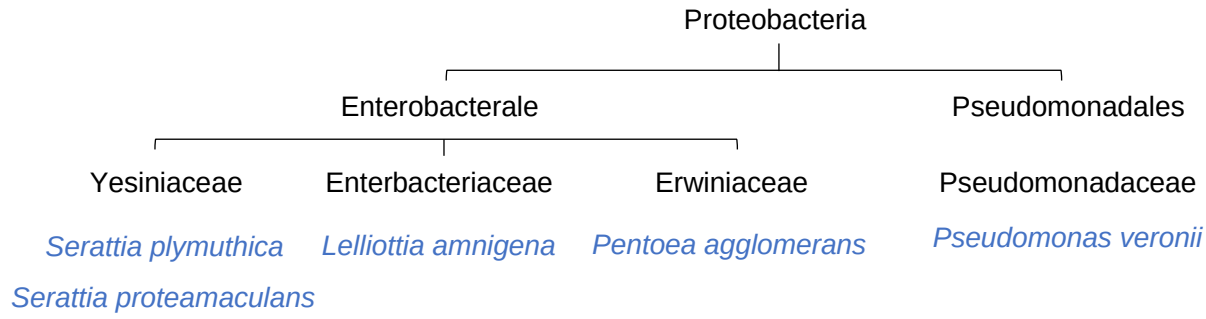


Figure 4.26. Simplified phylogenetic representation of identified isolates from the soil microbial community. 11 isolates from the soil communities were identified at the species level using MALDI-TOF MS. Isolates identified come from five different species, from four different families and two different orders. This is only a small sample of the true diversity present in the model soil community.

Initial growth experiments compared productivity (OD_{600}) under changing incubation conditions (static or shaken), period (days) and nutrients (media). A change in community productivity ($OD_{600,}$ Fiegna *et al.,* 2015) would indicate a selective effect. These results confirmed that O_2 limiting conditions in static microcosms produced lower community productivity than in shaking conditions where O_2 is homogeneous. Community productivity increases with incubation period, where between three and six days community carrying capacity was reached and productivity plateaued. Changes to productivity were also found between different media types, with minimal media supplemented with glucose demonstrated significantly lower productivity than KB* and LB.

KB* is a nutrient rich media, producing high community productivity and diversity based on colony morphology. It was important to select growth media capable of supporting the highest possible diversity, to improve the ecological relevance of the model community. This is also thought to provide more opportunity for interspecies interactions and coevolution, further enhancing the ecological relevance (Røder, Sørensen and Burmølle, 2016). However, KB* media is still likely to have a selective effect within the community, with complex high nutrient media likely selecting for fast growers. Changing incubation period

had a significant effect on community productivity, and from this result one, three and six-day incubation periods were selected for the serial-transfer experiments. Within 24 h the carrying capacity of the soil community is not reached and provides opportunity for adaption of faster growing community members. Between three and six days it was expected competition for limiting resources would increase (Bachmann *et al*. 2016; Hodapp *et al.* 2019) and total community productivity would also increase through adaption to changes within the system.

The initial development of a soil-wash model community in static liquid microcosms demonstrates a selective effect of an O_2 limiting environment and time limitations. KB* microcosms support a high level of diversity, and although the true diversity of a soil community cannot be replicated, these conditions provide a high starting diversity for serial-transfer experiments. The soil community consists of A-L interface biofilm competent strains that quickly establish an O_2 gradient in static liquid microcosms. The presence of aerobic species are likely to compete for the high-O_2 region below the A-L interface, while facultative anaerobes are presented with a likely trade-off between faster growth in the high-O_2 regions, or slower but less competitive growth in the low-O_2 liquid column.

4.3.2 Community productivity and biofilm-associated changes in serially-transferred communities and inhibition of maximum community productivity

O_2 gradients are ubiquitous in nature, and studies show adaption of single-species systems in response to O_2 limiting conditions (Koza *et al*., 2017: Kovács and Dragoš, 2019). However, the response of complex microbial communities to heterogenous O_2 limiting conditions is poorly understood. Large serial-transfer experiments were conducted to investigate the effect of O_2 limiting conditions and time limitation on the productivity and aggregated traits of A-L interface community biofilms using static liquid microcosms. Soil-wash inoculum was prepared and inoculated in KB* microcosm to begin short-term evolution experiments. Community or biofilm-only samples were serial-transferred for a total of 10 – 60 days, with one, three and six-day incubation periods between transfers. Evolutionary and ecological processes have been observed over relatively short time scales in other serial-transfer experiments (e.g. Kaltz *et al*., 2012; Lawrence *et al.* 2012; Fienga *et al*., 2015; Castledine *et al*., 2019), therefore changes were expected within the time periods set. Several transfer conditions favoured biofilm-formation (longer incubation period with biofilm sample transfer), and shaken microcosms act as a homogenous control where no O_2 gradient would establish. Each transfer represented a pulse disturbance or re-seeding event, where communities recover each transfer re-establishing biofilms in fresh microcosms. This

type of fluctuating environment is representative of many natural ecosystems and infection sites, where microbial communities a subject to regular disturbance. To determine the selective effects of O_2 limiting conditions, community productivity and biofilm-associated aggregates traits were compared between the initial soil-wash community and the final transfer communities.

I expected that community productivity would increase as soil communities adapted to conditions within the microcosms system, where resource competition would select for the most successful individuals. This would also reflect in an improvement of biofilm characteristics, with increased biofilm attachment and strength, as many community members compete for the high-O_2 region in static liquid microcosms through A-L interface biofilm-formation. This would suggest strong A-L interface biofilm formers would be selected, especially with longer incubation periods between transfers and when selection is occurring with the biofilm community only. Surprisingly, community productivity decreased within the three and six-day transfer experiments, however there was an increase in one-day transfer experiments. Biofilm attachment significantly increased between the initial and final microcosms in three-day experiments, and in static six-day experiments. There was also a significant increase in biofilm strength across all static transfers, but not in shaking conditions. Modelling analysis confirmed that incubation period and conditions (static or shaken) have a significant effect on community productivity. Similarly, biofilm attachment but not biofilm strength had a significant effect on community productivity, and the finding that community productivity is positively correlated with biofilm strength and attachment suggest communities growing faster produce stronger and better attached biofilms. In all modelling analysis sample type transferred (community or biofilm) did not have a significant effect on productivity, and little difference in biofilm attachment and strength were found between the static community and static biofilm transfers. This suggests similar functional and biofilm-associated traits are dominating the biofilm each transfer, whether isolates form the liquid column are present or not. However, OD_{600} productivity measurements are not sufficient to identify strains with increased or decrease EPS levels, therefore further viable counts and identification of EPS genes would be needed.

Differences in community replicates were found in modelling analysis, however the variation contributed was small at 7.2%. This is no surprise as ecological and evolutionary changes in bacterial communities are not just a result of deterministic processes, but random chance also plays a part in succession patterns (Brockhurst *et al.*, 2009). Divergence in replicate populations is known to occur in microbial microcosms experiments (Johansen *et al*. 2019).

A larger percentage of the population (1.6%) was transferred each time compared to other serial-transfer experiments (Lenski's *Escherichia coli* long-term evolution experiments transferred 1% of the population, Lenski *et al*., 1991). This was to avoid a bottleneck effect with only one or two strains dominating, however the transferring process can still introduce a source of drift and stochasticity to the population passed to the next microcosm (Van den Bergh *et a*l., 2018). Similarly, although samples were extensively vortexed prior to sampling, complete community homogeneity is not absolute which can introduce variation between replicate populations (Lanfear, Kokko and Eyre-Walker, 2014) and larger sections of EPS material not successfully broken by vortexing may also introduce variation in OD_{600} measurements between replicates.

Each transfer new niche space is created with fresh nutrients introduced and waste products removed. This would suggest isolates best able to compete for nutrients and O_2 within the system would succeed within the community, suggesting faster growing bacteria would increase overall community productivity. This is a common finding within serial-transfer evolution experiments, with many reporting an increase in productivity found in bacterial populations through mutants arising within the population with increased fitness (Lenski *et al.,* 1991; Rainey and Travisano, 1998) and in experimental communities through adaption and positive interactions such as cross-feeding (Lawrence *et al*., 2012; Fiegna *et al*., 2014). This was found in the one-day serial transfer experiments. Initial selection tests (Figure 4.3) suggest the carrying capacity of the system is not reached after one-day. Here, the system is likely able to support the succession of faster growing bacteria, with nutrients yet to become limiting, increasing overall community productivity in the one-day transfer experiments. However, a significant decrease in productivity is found in the three and six-day transfer experiments. This has been reported in laboratory populations where the build-up of toxic metabolites effect dynamics and fitness, resulting in a decrease in productivity (Travisano, 1997). Similarly, recent research comparing toxic production regulation in both fluctuating (serial-transfer) and stable environments concludes toxic regulation evolves under serial-transfer (Doekes, de Boer and Hermsen, 2019). Here, cells switch between fast-growing non-toxic phenotypes to slower-growing competitive toxic phenotypes in response to increasing cell density (Doekes, de Boer and Hermsen, 2019). Further exploration of ecosystem engineering through metabolic activity and competitive interactions was needed to understand the decrease in productivity found in the three and six-day transfer experiments (see section 4.3.3).

Bacterial attachment increased across many replicate final communities, in particular the three-day transfer communities. Diversification in populations incubated in static conditions have resulted in mutations elevating c-di-GMP which increase biofilm attachment in *P. fluorescens* SBW25 and *P. aeruginosa* PA14 (Batanki *et al*., 2007; Flynn *et al*., 2016) and enhances matrix gene expression in non-motile *B. subtilis* strains (Richter *et al*., 2018), and colonies appear wrinkled on plates. Wrinkled colony morphs were observed in some replicate three-day transfer communities (see Figure 4.11), suggesting mutants with elevated attachment or EPS production may be present, increasing overall community strength and attachment. Isolates with stronger attachment may also be selected within the community due to a fluctuating environment created through serial-transfer. This may be isolates producing quorum sensing signalling molecules which increases biofilm attachment, as demonstrated in *Burkholderia cenocepacia* K56-2 (Tomlin *et al.,* 2005) or the production and utilisation of extracellular DNA, shown to increase biofilm attachment in *P. aeruginosa* PA14 (Nivens *et al*., 2001). Attachment increased in the three-day shaking community transfer, likely through a tidal effect created by shaking incubation, causing cells to wash up and attach to the glass vile. Cells present at the top of shaking culture may still attempt A-L interface biofilm-formation, and floating biofilms were found in some replicate shaken community transfers.

Biofilm strength also significantly increased in both static transfer treatments. No significant increase was found in shaken transfer treatments, suggesting selection for stronger biofilm formers is a result of O_2 limiting conditions. As expected, the biggest increase in strength was found in the six-day static transfers, as longer incubation requires a more robust and stable biofilm to prevent community collapse. The positive correlation with biofilm productivity, attachment and strength is in agreement with individual isolate biofilm characterisation in previous work with soil isolates (Ude *et al*., 2006).

Changes in community productivity and biofilm-associated traits were clearly demonstrated in static liquid microcosms, where environmental heterogeneity limits access to O_2 within the system. This suggests changes will also be reflected at the individual isolate-level, gaining further insight to selective effects. The surprising decrease in community productivity found in the three and six-day transfers suggests further experimentation and understanding is needed to understand community dynamics within this system. This was explored by looking into the effect of toxic waste accumulation and competitive interactions with the community as a result of prolonged incubation between transfers.

4.3.3 Environmental modification and bacterial interactions prevent community from maximising productivity

Community productivity in soil-wash inoculated microcosms significantly decreased after serial transfer of 30 – 60 days, with three or six-day incubation periods between transfers. I expected to find increasing community productivity as a result of adaption and resource competition within the system, selecting for faster growing community members as reported in previous serial-transfer experiments (Lawrence *et al*., 2012; Fiegna *et al*., 2014). Research which reportt a decrease in productivity suggest the build-up of toxic waste products, nutrients depletion and/or the development of competitive phenotypes limits community productivity (Travisano, 1997; Doekes, de Boer and Hermsen, 2019). Niche-construction through O_2 depletion is apparent within static liquid microcosms (Koza *et al*., 2017) however ecosystem engineering through metabolic activity may also have significant effect, depleting nutrients and the accumulation of waste products and metabolites (McNally and Brown, 2015). This can be linked to interference competition, through the active secretion of toxic waste products to compete for limiting resources. Ecosystem engineering and competitive interactions were explored using the six-day transfers communities to determine why productivity was decreasing.

Aged media was created by the growth and removal of community samples for one, three and six days, and community productivity was compared in fresh nutrients and the depleted environments. The aged media was used to dilute fresh KB* samples and compared to KB* diluted with water, where lower productivity in KB* diluted with aged media rather than water only would suggest a combination of nutrient depletion and waste product accumulation is creating a growth limiting effect. Finally, strains from the six-day transfer communities were tested for neutral or competitive interactions in spot-on-lawn interaction assays against isolates from the initial soil-wash. The presence of competitive or antagonistic interactions would further suggest toxic molecules being released into the environment through interference competition, further decreasing productivity.

Aged media experiments confirmed a negative effect on community productivity, with six-day aged media causing the biggest decrease in productivity (Figure 4.8). Fresh microcosms diluted with aged media decreased productivity more than dilution with water, until a concentration of x 0.1 where insufficient nutrients were present to support substantial community growth. Spot-on-lawn interaction assays confirmed the presence of competitive and antagonistic interactions in the six-day communities, with the static biofilm and static

community transfers containing more competitive isolates than the shaken community transfer. This suggests a static heterogenous environment created by O_2 limitations selects for more competitive phenotypes than a homogenous shaking environment.

Increased incubation periods provide longer time for nutrient depletion and waste product and secondary metabolite accumulation. Within the three and six-day community experiments the community carrying capacity is reached, as suggested by the initial growth comparison experiments (Figure 4.3). Communities have entered stationary phase, where nutrient become a limiting factor waste products dominates the environment. In evolution experiments this is often termed a 'feast-and-famine regime' or 'seasonal environment' (Vasi, Travisano and Lenski, 1994) and the accumulation of toxic waste products have been found to inhibit productivity in laboratory bacterial populations (Travisano, 1997). I suggest that the occurrence of a feast-and-famine regime in the three and six-day serial-transfer experiments acts as a further selective pressure, and more competitive phenotypes are selected. This likely results in the active secretion of toxins through interference competition for resource competition (Friedman and Gore, 2016). This suggests a selection for slower-growing competitive phenotypes in response to increased cell density, found in fluctuating environments with high cell density (Doekes, de Boer and Hermsen, 2019). To confirm growth curve comparisons between initial soil-wash isolates and final transfer community isolates is needed to determine differences in growth rate. Further community development is hindered, characteristic of a tragedy-of-the commons colonists inhibit the development of future community members (Maclean, 2008). Future work would also test isolate supernatant for the presence of antimicrobial products, and aim to identify key waste products and toxic metabolites through metabolomics (Tyc *et al*., 2017) and determine if these are actively secreted toxins in competition, anti-completive compounds (Hibbing *et al.*, 2010) or metabolic waste products (Maclean, 2008).

Although competitive interactions were found, communities were still dominated with neutral interactions. To further explore the presence of neutral interactions within the community, productivity was compared between mixed strain microcosms with one to eight strains, and the corresponding community sample. No significant differences were found in the one and three-day biofilm transfer community samples, confirming neutral interactions between community members. However, there are individual examples of synergy and competition within these communities. In the one-day biofilm community a dual-strain combination produced lower productivity than the single strain culture, suggesting a competitive relationship or cheating between the two strains (Griffin, West and Buckling, 2004). In the

three-day biofilm community, a single isolate exhibited very low productivity in monoculture, but productivity is higher when placed in a dual-isolate culture, suggesting a commensalism or mutualistic relationship between the two isolates such as cross-feeding (D'Souza *et al*., 2018). This confirms the complex network of interactions within a community, and interaction type is likely due to specific strain relationships (Ren *et al.,* 2015) rather than a trend in diversity-richness relationship. Within the six-day biofilm transfer community, productivity in the community sample is significantly lower than combinations of two to eight isolates. I suggest this is caused by an increase in competitive phenotypes found during the spot-on-lawn interaction assays, causing further resource competition and selection for slow-growing toxin producing strains (Doekes, de Boer and Hermsen, 2019). These experiments were conducted with isolates from the same community, therefore interactions could be influenced by co-selection. Further experiments mixing isolates from different communities would be interesting to reveal further insight into the type of social interactions of these isolates. This research concludes that complex ecological dynamics including ecosystem engineering and competitive interactions shape community productivity in A-L interface biofilm-forming communities.

4.3.4 Individual isolate-level analysis reflects changes found at the community level and reveals further selective changes in serial-transferred communities

Productivity and biofilm-associated traits were significantly affected by serial-transfer in static liquid microcosms. Individual strain traits are known to shape the emergent function of microbial communities (Konopka, 2009), suggesting further changes can be found at the individual isolate-level. Further characterisation of individual isolates such as behavioural and functional traits can provide further evidence and patterns in community change. The combined biofilm assay was used to characterise A-L interface biofilm-formation at the individual isolate-level, and a series of phenotypic and behavioural assays were measured to capture trait-based changes. It would be expected that changes in biofilm characteristics found at the community-level would be reflected at isolate-level. I also expected to see changes in the behavioural and phenotypes of strains between the initial soil-wash and final communities, and between the final communities as static conditions will pose different selective pressures. Similarly, longer incubation periods and evidence of ecosystem engineering may select for nutrient scavenging behaviours such as siderophore production.

Isolate-level biofilm characterisation confirmed reflected changes found in community biofilm properties, with the greatest shift in biofilm phenotype space found in the six-day static community and static biofilm transfer. This also allowed for Odds ratio calculations, indicating A-L interface biofilm-formation was 4 – 7 x more common in the six-day static transfers than the initial soil-wash. This indicates prolonged incubation in O_2 limiting conditions selects for A-L interface biofilm-formation. It also suggests communities have retained a degree of functional redundancy, with most members capable of A-L interface biofilm-formation.

Phenotype and behaviour assays also confirm significant difference between the initial soil-wash and the final communities. Oxidase and catalase production is a common test combination for species or subspecies identification, with results indicating the presence of aerobic bacteria through oxidase production confirming utilisation of O_2 as an electron acceptor, and the ability to neutralise toxic forms of O_2 through the production of catalase. Oxidase production was selected in the one and three-day shaken communities. This suggests homogenous conditions with high-O_2 levels and shorter incubation periods select for fast-growing aerobic bacteria. This was also found in the six-day static community and static biofilm transfers, where more A-L interface biofilm-formers are found indicating an association with A-L interface biofilm-formation and oxidase producing strains. Similar patterns in catalase production were found with selection for this trait in the six-day static community and biofilm transfers. Previous experiments demonstrate waste product accumulation in six-day transfer experiments. Dominance of catalase production in the six-day static communities suggest prolonged conditions selects for strains capable of neutralising toxic O_2 metabolites. Similar results are found in the three-day shaking community, but selection for catalase production is not found in the one-day shaken community, suggesting this incubation period is too short for toxic O_2 metabolites to build-up.

Bacteria, typically aerobic strains, produce siderophore molecules to chelate iron under limiting conditions (Neilands, 1981). Although siderophore production is common in pseudomonads (Cornelis, 2010) it is a common trait amongst many species including *Streptomyces, Staphyloccoccus, Serratia* and *Azotobacter*, and strains of *Escherichia coli, Bacillus anthrasis* and *Vibrio cholera* (Glick 1999; Saha *et al*., 2013). Significant selection for siderophore producing isolates were found in the six-day static community, indicating prolonged incubation creates resource heterogeneity as well as an O_2 gradient. All shaken transfer experiment show significantly less siderophore producing isolates compared to the initial soil-wash, suggested there is still sufficient free iron in the environment after six-days

and siderophore production is selected against in homogenous environments. This may indicate free iron is still available under static conditions but is depleted in the high-O_2 region where competition is higher and cells become sessile within the biofilm through EPS production limiting movement towards fresh nutrients (Ha and O'Toole, 2015). This would suggest an opposing nutrient gradient, with low nutrients and free iron with the high-O_2 region of static liquid microcosms, and more available nutrient within the liquid column where cells present can utilise energy-sensing taxis throughout the liquid column to obtain nutrients. Siderophore production is also thought as a social trait, and mutants of *P. aeruginosa* PA01 deficient in cooperative siderophore production were deficient in biofilm-formation (Harrison and Buckling, 2009), which may also explain the increased siderophore production in six-day static conditions. Similarly, resource heterogeneity has been shown to evolve cooperative siderophore production, compared to homogeneous environment where cheating phenotypes develop (Stilwell *et al*., 2020). This would suggest shaking incubation creating resource homogeneity results in selection for cheating phenotypes not producing siderophores, and static microcosm transfers where resource heterogeneity develops selects for cooperative siderophore producing phenotypes. Siderophore cooperation and cheating usually is usually amongst individuals closely related which share the same siderophore system (Kramer, Özkaya and Kümmerli, 2020) suggesting final transfer communities could contain closely related species allowing siderophore interactions to develop.

Isolates from the six-day transfer community also show increased foam-production, suggesting production of a surfactant-like molecules, capable of lowering surface tension. Chapter 3 demonstrated a key step to successful colonisation of the high-O_2 region is interacting with A-L interface through surface-active agents, lower surface tension and position cells above the A-L interface. As demonstrated, the six-day static transfers selected for more biofilm-formers and competitive phenotypes. I predict that the ability to produce surface-active or organic molecules lowering surface tension (Kjelleberg, 1985) is advantageous when incubation periods are extended and competition for the high-O_2 region is high. O_2 gradients establish within biofilms (Wessel *et al*., 2014) and the ability to further break surface tension positioning cells above the interface in contact with air would be advantageous for aerobic species. Cells within the biofilm or unable to break surface tension may be positioned under the interface or pushed down within the biofilm where O_2 is lower. Future work would aim to characterise surface-active properties of community isolates through tensiometer analysis, and further characterise if the ability to lower surface tension

provides A-L interface biofilm-forming strains with a competitive fitness in a community of similarly capable strains.

The biofilm and phenotype data was utilised to calculate species diversity using a dominance (Simpson reciprocal) and information-statistics (Shannon) indices. Here the Simpson's reciprocal Index gives the probability of two individuals being from the same species, so is weighted towards species abundance. The Shannon index includes rare species in the community and is affected by species evenness. There was a decrease in diversity found in all final transfer communities, with the biggest decrease found in the six-day static community and static biofilm transfer, which reflects the significant changes found in biofilm and phenotypic traits. This also confirms that prolonged growth in O_2 limiting conditions has a significant effect of the functional diversity and traits within a biofilm-forming community. A large decrease in biofilm and phenotypic diversity was also found in the one-day shaking community. This indicates a homogenous environment with short growth period between disturbances has a strong selective effect, and previous phenotypic and behaviour characterisation suggests selection for fast growing aerobic species. There is a larger diversity found in the shaking environment with prolonged incubation, perhaps supporting slower growing species and alternate functional traits such as biofilm-formation.

Species indices and productivity data was utilised to explore the relationship between diversity and productivity. The productivity-diversity relationship is complex (Smith, 2007) with positive, negative and unimodal relationships found (Mittelbach *et al*., 2001). There was no significant correlation found between community productivity and richness, calculated using community productivity and species indices data. Further 16S rRNA amplicon and metagenomic sequencing would be needed in future work to explore the relationship between productivity and richness and diversity and uncover further trends and changes in community aggregated traits (Fierer *et al*., 2014).

The trait-based approach for quantifying changes in diversity is a promising experimental approach in the future of microbial research. In the last two decades there has been a shift in ecology for biodiversity-ecosystem functioning research using successful trait-based approaches (Krause *et al*., 2014). Within this research two common species indices were used to give a broad overview of the changes in trait diversity found within serial-transferred communities. Both have limitations, however, provide overall trends and address the whole distribution of the sample (Hill *et al*., 2003). Many trait-based indices are being developed to describe community changes (Fontana, Petchey and Pomati, 2016), an interesting future

approach to compare and contrast with widely used dominance and information-statistic indices. Recent research characterising morphophysiological traits of lake microbial communities improved predictability of community resource-use and biomass yield compared to taxonomic characterisation, suggesting individual-level trait characterisation improves our ability to predict changes in ecosystem properties across environmental gradients (Fontana *et al*., 2018). Future work would aim to explore newly developed trait indices and compare to traditional dominance and information-statistics indices. It would also be interesting to compare functional and trait-based diversity with taxonomic diversity, measured through 16s rRNA sequencing and metagenomics (Goldford *et al*., 2018). Here the link between species and functional diversity could be explored, as evidence suggests species can exhibit different functional traits and behaviours dependent on environmental conditions (Kraemer *et al*., 2010 and Vos and Vellicer, 2006). While recent evidence suggests functional diversity and genetics are not necessarily mapped (Kraemer *et al*., 2010 and Vos and Vellicer, 2006) and ideal study would encompass both functional and genetic changes, furthering our understanding of the relationship between genetic and trait-based changes.

Further analysis at the individual-isolate-level confirms changes in biofilm-associated traits between communities undergone serial-transfer in O_2 limiting environment. Phenotype characterisation identified key social and behavioural traits favoured in both static and shaken conditions, demonstrating selection within the system. Further evidence of selection was confirmed by diversity indices, using a trait-based approach to quantify diversity. There were significant difference in diversity between communities, however functional redundancy is still present, with many isolates capable of similar functional traits, in particular A-L interface biofilm-formation.

4.3.5 Community stratification and retention of liquid column productivity maximises total community productivity and selects for phenotypic plasticity

While the high-O_2 region was colonised through biofilm-formation between transfers, and most isolates were capable of biofilm-formation, the lower liquid column was also re-colonised each transfer. Similarly, during isolate-level analysis most isolates from final transfer communities were capable of A-L interface biofilm-formation but also retained population growth in the liquid column. This was surprising in the biofilm-only serial-transfer experiments, where cells from only the biofilm were transferred, where I expected

communities to be dominated with strong biofilm-formers with little liquid column growth. In static liquid microcosms the low-O_2 liquid column consists of >90% of the environment (Koza *et al*., 2011). Although the liquid column is depleted in O_2 after initial growth, there is still a rich source of nutrients within the media. The cell localisation assay developed in Chapter 3 provided a unique insight into productivity below the A-L interface biofilm in the *P. fluorescens* SBW25 model system, and its influence on strain fitness. A key knowledge gap in biofilm research is the influence and productivity of neighbouring non-biofilm space, where planktonic cells can be in close contact with the biofilm community. The cell localisation assay was further developed to determine productivity throughout the entire community, where the biofilm and liquid column community could be compared. Swimming motility was characterised and transplant experiments were designed to determine if cells within the liquid column interact with the biofilm community, and vice versa, suggesting cells can migrate between both regions. During investigation of liquid-column productivity, a more focussed approach was taken, utilising the initial soil-wash and the six-day transfer communities. The six-day static communities demonstrated the greatest changes suggesting a high degree of selection had taken place, therefore changes would also be expected in the liquid column.

Cell distribution experiments confirm productivity heterogeneity throughout, and as expected community samples resulted in a stratified community, with A-L interface biofilm-formation, and planktonic growth in the liquid column. The cell localisation experiments confirmed that productivity was retained in the liquid column of final transfer communities, however static transfer treatments where O_2 gradients can develop result in higher productivity in the A-L interface biofilm community. This is in agreement with the finding that isolates from the static transfer treatments have altered biofilm characteristics, and that more biofilm-forming isolates were found in these communities. Higher levels of enrichment at the top of the liquid column in final transfer communities compared with the soil-wash inoculum suggests selection for strains better able to compete efficiently for the high-O_2 niche. However, ecological filtering was not strong enough to reduce colonisation of the low-O_2 region, despite the fact that biofilms are known to steepen the O_2 gradient formed in static microcosms (Koza *et al.* 2011; Loudon *et al*. 2016). Total productivity in the mid-column region (pooled across samples) was significantly greater than at the top (1.9 – 3.8x; Figure 4.6), demonstrating that the larger low-O_2 region dominates productivity in community microcosms. However, productivities were lower for the six-day static biofilm community than the static community transfer, which may reflect the gradual loss of mid-column strains or migrants during the serial transfers of biofilm-only or mixed-community samples. It also

indicates within the static biofilm transfer community, members were not equally distributed between the high and low-O_2 regions of static microcosm. However, higher cell localisation at the A-L interface could potentially further blocking O_2 from diffusing into the system. This could further steepen the O_2 gradient within these systems, supporting lower growth in the liquid column. Future working would aim to characterise O_2 gradient in different communities through O_2 profiling (Koza *et al*., 2011) to determine if higher localisation to the A-L interface effects the O_2 gradient within the system, or if biofilm-formation alone is sufficient in locking O_2 access, relevant of cell density. Findings from cell localisation experiments suggest physiological plasticity may play an important role in allowing serial-transfer communities to maximise productivity by colonising both regions of static microcosms (Beier *et al*., 2015), and retaining generalist phenotypes within the community (Van ben Bergh *et al*., 2018).

Swimming motility was common amongst community isolates, confirmed by swimming motility assays. Non-motile strains were only found in the initial soil-wash and 6-day shaken community. Static incubation with O_2 limiting conditions selected against non-motile strains, while non-motile strains were found in the initial soil-wash and six-day shaken community only. In shaken conditions no O_2 gradients is established, eliminating competition for niche space. Similar findings were shown in static cultures of *P. aeruginosa* FRD1 which are quickly dominated by alginate-producing mutants that acquire flagella motility in response to O_2 gradient sensing, while typical mucoidal *P. aeruginosa* FRD1 shaking cultures have no motile cells (Wyckoff *et al*., 2002). Motility is required to access the high-O_2 in static liquid microcosm, and also provides opportunity for cells to migrate between regions. Transplant experiments confirmed that community members present in the liquid column could colonise both the liquid column and form A-L interface biofilms in fresh microcosms, and vice versa with samples taken from the biofilm community. The source of the transplant sample did not significantly affect productivity, suggesting strains can maximise total microcosm productivity, regardless of where they were situated at the time of sampling. There were changes in biofilm strength, indicating stronger biofilm-forming isolates dominate the biofilm community, compared to the liquid column. Comparison of growth under varying oxygenation conditions confirmed that community isolates reached maximum productivity under high-O_2 conditions, suggesting the re-establishment of growth in the lower liquid column each transfer was not a result of obligate anaerobes.

Isolates in final transfer communities were capable of A-L interface through biofilm-formation and planktonic growth in the low-O_2 liquid column, and most were motile. This suggests serial-transfer in O_2 limiting conditions selects for high levels of phenotypic plasticity, allowing

communities to maximise productivity by colonising both regions and migration between the two coalescing communities. Migration experiments were run for sufficient time for cells to localise and migrate to preferred region within the system, as demonstrated by earlier cell localisation experiments within the *Pseudomonas fluorescens* SBW25 system, where a microcosms with uniform cell distribution quickly results in layering with cell localisation dominating the A-L interface. It would be interesting to determine species composition of each fraction in future work, to determine how diverse each region is, and if the same strains are present throughout showing flexibility. It should be noted that in these tests migration and growth are linked but as the random diffusion of cells is so slow, the presence of cells in both regions is evidence of migration. Similarly, although it is presumed that most cells found mid-column are free swimming 'planktonic' cells, this region may also contain sinking biofilm fragments, as bacteria cells grow in small aggregates even in liquid culture (Schleck et al. 2009). Plastic phenotypes, similar to generalist phenotypes (Van den Bergh et al., 2018), can occupy the high-O_2 region through biofilm-formation, or growth in the liquid column where O_2 is limiting. Cells are likely presented with a resource-allocation trade-off (Arcerenca, 2016; Ferenci, 2016), but the ability to occupy both niches presents a fitness advantage in serially-transferred biofilm communities.

Biofilm-formation at the A-L interface is initially costly, but strains benefit from increased growth rates through high-O_2 conditions increasing fitness within the community. However, this niche makes up a very small area of the microcosm environment, so competition for this niche and with biofilm community is high. This was confirmed by more competitive phenotypes found in the static transfer regimes (six-day static community and six-day static biofilm transfer). Nutrient gradients likely develop, as evidenced by selection for siderophore producers in static transfers, indicating cells need nutrient scavenging molecules to adapt and succeed (Saha *et al*., 2013). Chemical heterogeneities through metabolic activity and solute diffusion are known to establish within biofilms (Stewart and Franklin, 2008) and gradients are likely steep in six-days of static incubation. Cell movement within a biofilm is restricted by the biofilm matrix, and motility is often switched off to turn on EPS production (Ha and O'Toole, 2015). This will likely lead to quicker depletion of nutrients and cells are at risk of exposure of toxins produced through interference competition by neighing strains (Stubbendieck and Straight, 2016). Within the liquid column, strains are faced with slower growth rates under low-O_2 conditions, however even strict aerobes can still maintain growth until O_2 levels are very low (Couvert *et al.* 2019). The liquid column is a larger environment than the high-O_2 region, making up more than 90% of the environment (Koza *et al*., 2011). O_2 levels are low so cells are faced with slower growth rates. However, nutrient levels are

likely higher, and competition less prevalent. Cells can utilise energy-sensing taxis to obtain further nutrient throughout the liquid column and move away from toxins and metabolic waste products. Similarly, random diffusion and further convection movement created by Brownian motion and bioconvections currents allows nutrients and metabolites to move throughout the large liquid column. Cells therefore are not subject to harsh chemical gradients found within biofilms (Stewart and Franklin, 2008).

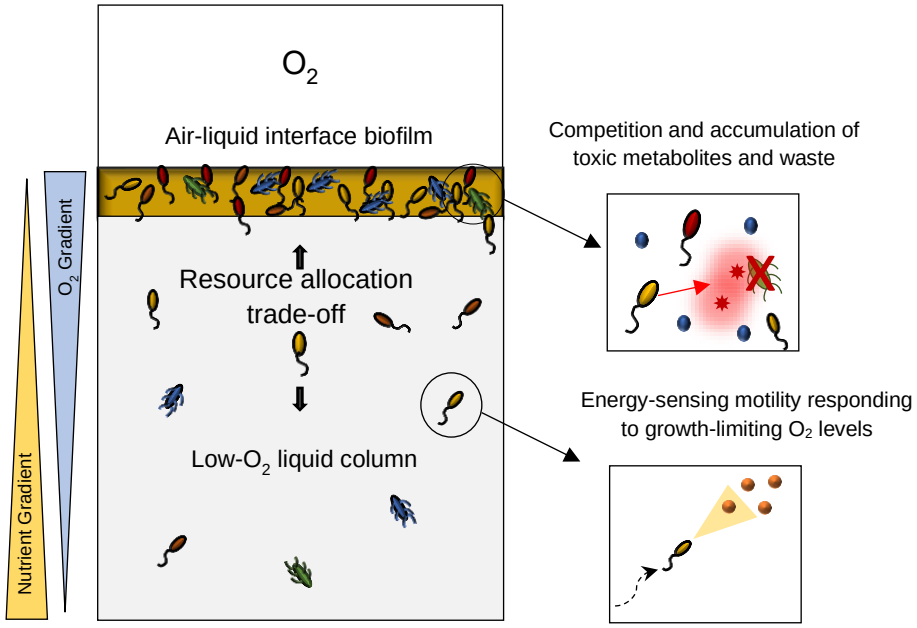


Figure 4.27. Maximising community productivity with plastic phenotypes retained in serially-transferred static liquid microcosms. Bacterial strains surviving in community microcosms are faced with a resource allocation trade-off, as metabolic activity included in the uptake of O_2 and nutrients result in opposing gradients. Cells can form A-L interface biofilm in the high-O_2 region which increase growth rates. However, the high-O_2 region is a competitive niche, with nutrient heterogeneity, waste product accumulation and competitive phenotypes releasing toxins through interference competitions. Cells in the liquid column are subject to O_2 limiting conditions slowing growth rate however, through energy sensing motility can acquire nutrients and avoid toxic waste products.

A serial-transfer approach creates a fluctuating environment, where communities are disturbed, leading to constant changes in O_2 conditions and nutrient availability. Fluctuating environments are predicted to select for generalists or plastic phenotypes (Pigliucci, 2001; Van den Bergh *et al.,* 2018) explaining the retention of plastic phenotypes during serial-transfer experiments. Plastic phenotypes can survive in both O_2 and nutrient depleted

conditions, and form biofilms at the A-L interface to access the high-O_2 region. This may also be considered as a bet-hedging approach, as isolates split the population into biofilm-formers and planktonic swimming cells. Biofilm-formers have increased access to O_2, but this highly competitive niche will result in further nutrient gradients and strains are at risk of exposure of toxins produced through interference competition by neighing strains (Stubbendieck and Straight, 2016). Planktonic cells remaining in the liquid column are in sub-optimal O_2 growth conditions, however can easily avoid competition and locate nutrients through energy-sensing motility. In the event of biofilm collapse a large proportion of the population remain within the liquid column and can even re-establish the biofilm.
This work suggests that studies of community-based (multispecies) biofilms should include the surrounding non-biofilm space to better understand the impact on biofilm–formation and persistence by migration of bacteria with plastic phenotypes. In this context, bacterial migration might have serious consequences for the control and elimination of pathogens in medical, veterinary and agricultural contexts, and in the control and maintenance of communities used in food production and other industrial biomass-conversion processes. Future work would look to follow individual community members through neutrally marking community member through a reporter gene approach of utilising in situ hybridisation to visualise and confirm the ability of migration between the liquid column and biofilm community.

4.3.6 Communities selected under O_2 limiting conditions are more resistant to invasion in static liquid microcosms

Community succession resulting in changes in diversity and development of interactions can affect community resistance and resilience, in particular community invasability (Jiang and Morin, 2004). Studies of community resistance and resilience suggest more diverse communities are likely to contain taxa with an appropriate trait response increasing community resistance, known as the insurance hypothesis (Yachi and Loreau, 1999) Similarly, communities with a high level of functional redundancy also increase community resistance and ability to recover after disturbance (Allison and Martiny, 2008). The six-day serially transferred communities show significant changes in community diversity, with the static community and static biofilm transfers demonstrating the lowest diversity. Invasion experiments where designed to test if the loss in diversity increased susceptibility to invasion, or if adaption to the O_2 limiting environment and increased competitive phenotypes were more important in protecting communities against foreign species. Marked strains of *P. fluorescens* SBW25 wild-type and Wrinkly Spreader mutants were introduced into cultures of

the initial soil-wash and six-day transfer communities using a reporter gene assay. This allowed community invasion experiments with both a strong A-L interface biofilm former (WS mutant) and a strain capable of growth in the liquid column (wild-type SBW25).

SBW25-*lac*Z (Zhang and Rainey, 2007) was utilised to construct WS-*lacZ,* and both strains produced blue colonies on plates supplemented with X-Gal so could be distinguished from the community isolates (Figure 4.23). The marked invading strains were tested for neutrality, and the WS-*lacZ* has a small significant increase in fitness. However, as WS-*lacZ* represents an invading strain, an increase in fitness compared to the archetypal WS was not a concern and provided a more competitive invader. The invading strains were incubated in pair-wise fitness assays with the four test communities, in both static and shaken conditions. Pair-wise competitive fitness competitions revealed that SBW25-*lac*Z and WS-*lacZ* were unable to invade the six-day static community and static biofilm community, with almost complete inhibition of the invading strains. Under shaking conditions, all communities were resistant to invasion, however the WS-*lacZ* mutant successfully invaded the six-day shaking community in static conditions. The fitness of SBW25-*lac*Z was equal to the six-day community and the WS-*lacZ* mutant to the initial soil-wash community under static conditions. Results under static conditions suggest communities undergone selection in O_2 limiting conditions are better adapted to the environment and more resistant to invasion. However, CFU counts of the invading strains were very low, suggesting a further inhibitory effect preventing growth of the invading strain. To investigated, spot-on-lawn interaction assays were carried out with community isolates and the invading strains. This confirmed significantly more isolates from the six-day static community and six-day static biofilm community displayed competitive or antagonistic interactions towards both SBW25-*lac*Z and WS-*lacZ,* suggesting competitive phenotypes increase community resistance to invasion.

The insurance hypothesis suggests that more diverse communities are more resistant (Yachi and Loreau, 1999), but here more diverse communities (initial soil-wash and six-day shaken community) performed the poorest in invasion experiments. This contrasts to the negative correlation between soil microbiota diversity and survival of *E. coli* O157:H7 when invading (Dirk van Eslas, 2012). However, while the static communities have significantly lower diversity, there still appears to be a high level of functional redundancy (Allison and Martiny, 2008), with many isolates performing similar traits such as biofilm-formation and production of nutrient scavenging molecules. A high level of functional redundancy is found in soil isolates, and even when a soil microbial composition is altered through dilution, no functional changes are found in microcosms (Wertz *et al*., 2007). Retention of functional redundancy is

important for community recovery and resistance to invasion. As these communities retained plastic phenotypes occupying both the A-L interface through biofilm-formation and growth in the low-O_2 liquid column, communities were resistant to invasion from both strong A-L interface biofilm-forming mutants (WS mutant) and a strain capable of growth in the liquid column (wild-type SBW25). The static communities also had a higher level of competitive and antagonistic interactions. Interactions have been previously shown to be important in resistance against invasion, with models of soil isolates showing synergistic interactions between community members increases resistance (Burmølle *et al*, 2006), and competitive interactions can alter the diversity-invasion relationship (Yang *et al*., 2017). As competitive interactions can be strain specific, future work would at repeating invasion experiments, along with spot-on-lawn interactions assays, to determine if this competitive mechanisms of resisting invasion is specific to *P. fluorescens* SBW25, or applied to other invading species.

4.4 Conclusion

Throughout this chapter I have developed a multi-species model system using soil-wash inoculum to investigate the effects of a heterogenous O_2 limiting environment in static liquid microcosms. During serial-transfer of a soil microbial community productivity and biofilm associated traits fluctuated, but each transfer A-L interface biofilms were re-established in static conditions. After ten transfers biofilm-associated traits were significantly altered, with the 30-day experiment with three-day transfers selecting for higher levels of biofilm attachment and the 60-day experiment with six-day transfers selecting for stronger biofilm formers. This was reflected in a higher proportion of community members capable of biofilm-formation. Productivity surprisingly decreased in the three and six-day transfer experiments, and I conclude this is a result of ecosystem engineering creating a tragedy of the commons effect through depleted conditions, and selection for more competitive phenotypes capable of interference competition through release of toxins. Interactions within the communities are however a balance of competitive, synergistic and neutral. Further investigation revealed changes at the community level were reflected at the individual strain level with altered biofilm characteristics and phenotypic and behavioural traits.

Productivity in the low-O_2 liquid column still dominated community productivity, even when selection favoured biofilm-formation. Transplant experiments and motility characterisation suggests serial-transfer selects for phenotypic plasticity, where isolates can form A-L interface biofilms and colonise the liquid column with migration between both niches. This

suggests a resource allocation trade-off between fast but more competitive growth at the A-L interface where further resource gradients develop within the biofilm, and slow but less competitive growth in the low-O_2 liquid column. This highlights complexity in community-based biofilms and the influence of surrounding non-biofilm space. My research suggests non-biofilm space can strongly interact and influence the impact and persistence of biofilms and may have a serious consequence when eliminating pathogens in medial and food industry settings or help control and mediate communities for industrial and agricultural use and for biomass-conversion.

Seminal analysis from this work contributed to ideas and area discussed in our book chapter 'Extending an eco-evolutionary understanding of biofilm-formation at the air-liquid interface to community biofilms' published in InTech's publishing's *Bacterial biofilms* in October, 2020 (Appendix 2). The main results from this chapter formed the publication 'Community biofilm-formation, stratification and productivity in serially-transferred microcosms' published in FEMS microbial letters in December, 2020 (Appendix 2).

Chapter 5. Understanding ecosystem engineering in the *Pseudomonas fluorescens* SBW25 model system

Abstract

Environmental modification through ecosystem engineering is commonly observed within ecological studies. Basic biological processes, such as metabolic activity which utilises nutrients and releases secondary metabolites and toxic waste products, produce an altered chemical environment which may no longer favour the growth of the original colonists but may provide an ecological opportunity for novel adaptive mutants and lineages. Ecosystem engineering and niche creation have been explored using *Pseudomonas fluorescens* SBW25 in static microcosms, where the uptake of O_2 in the liquid column by the rapidly growing population creates a relative high-O_2 region directly below the A-L interface which provides an ecological opportunity for the Wrinkly Spreader adaptive mutants. Here the impact of aging media on WS evolution, phenotype (biofilm characteristics and colony morphology) and fitness was explored. Significant chemical changes were found in aged media (spent media with prolonged growth period allowing for nutrient depletion, and waste product and metabolite accumulation) produced by wild-type SBW25 colonists, including pH, extracellular DNA levels, and FTIR spectra. The aged media had an inhibiting effect on the growth of both wild-type SBW25 and the archetypal WS mutant cells, characteristics of a tragedy of the commons effect. The numbers of WS mutants arising within wild-type SBW25 populations, and biofilm characteristics of WS mutants grown in aged media were also significantly affected. Diversification and fitness were further explored in evolution experiments using one-day aged media. Aged media WS mutants produced weaker biofilms than the archetypal WS mutant, but had greater fitness in static microcosms with both fresh and aged media compared to the archetypal WS mutant. This suggests that diversifying wild-type SBW25 populations are altering their chemical environment and these changes are

selecting for different WS mutants which are better adapted to the chemical changes. These mutants are still able to access the high-O_2 region of static microcosms through biofilm-formation, where they have a fitness benefit compared to other non-biofilm–forming competitors. This research provides further insight into the ecological dynamics of static liquid microcosms, where ecosystem engineering by the initial colonists, as well as the diversifying population, constantly modifies the chemical environment and alters the selective pressure on adaptive lineages.

5.1 Introduction

Microorganisms chemically and physically modify the abiotic environment affecting residing species, and can have both positive or negative effects. The concept of environmental modification is used both in ecology and evolutionary biology, with two similar terms used to describe this process. Within evolutionary biology environmental modification is often termed 'niche-creation', where organisms significantly modify the environment effecting the selection pressures within the system resulting in an evolutionary response within recipient organisms (Mathews *et al*., 2014). The term niche-construction is now recognised as a key evolutionary process, often resulting in adaptive-radiation in microbial populations (Schutler, 2000; Koza *et al*., 2017). Many conclude a central concept of niche-construction is the ability of the modifying species to better respond and outcompete neighbouring species in response to the environmental change (Callahan, Fukami and Fisher, 2014). Within ecology a broader definition is more often used, termed 'ecosystem engineering', where direct or indirect alteration of the physical or chemical environment occurs by modification, maintenance or creation of habitat (Jones, Lawton and Shachak, 1994). However, both draw on key aspects of physical or chemical modification resulting in positive or negative effects on the residing population and community.

Ecosystem engineering and niche-construction are relatively new concepts within the field of ecology and evolutionary biology and most research focuses on macrosystems, e.g. the creation of shade by plants and the creation of habitat by animals (Erwin, 2008). Microbial examples are less prevalent, but many accept the ability of environmental modification by microorganisms needs to be taken into consideration in microbiome health and infectious disease studies (Esterla, Whiteley and Brown, 2014). A key and common process in which microorganisms can alter the external environment is through metabolism. Bacteria can alter the chemical environment through the uptake of limiting resources within the system, and production of metabolic by-products, soluble enzymes (e.g. proteases), scavenging molecules (e.g. siderophores), antibiotics and detoxification products (McNally and Brown, 2015). This also includes secondary metabolites in pathogenic species, increasing virulence of the engineering species. Clinical isolates of *Pseudomonas aeruginosa* produce the secondary metabolite pyocyanin, able to oxidise and reduce many other molecules. This metabolite is toxic to humans and other microbes, killing competitors and increasing the fitness of *P. aeruginosa* (Lau *et al*., 2004). Many species, in particular pseudomonads, produce molecules to increase nutrient uptake and fitness. Siderophores are released for the essential uptake of iron (Cornelis, 2010). However, the production of siderophores creates an iron-limiting environment, impacting other cells which may have to adapt and alter

metabolism to survive. Metabolic and scavenging processes are common in microbial populations and communities, but the resulting ecosystem engineering effect on the environment and neighbouring cells or future lineages remains unclear.

A key environmental consequence as a result of metabolic activity is the change of pH (Ratzke, Jonas and Gore, 2018). Small changes in pH can cause an extreme negative effect on the growth and survival of bacteria, even resulting in the extinction of a population. This is a phenomenon sometimes termed 'ecological suicide' or when the effect is lesser, 'self-inhibition' (Ratzke, Jonas and Gore, 2018). Microbes have an optimum pH for growth, and above or below this can cause cell death or growth inhibition (Jones *et al*., 2009; Russell and Dombrowski, 1980). pH can be considered a 'public bad' (as opposed to 'public goods') as it is produced collectively by members, and harms all members of the population (Ratzke, Jonas and Gore, 2018). This was recently demonstrated in populations of *Paenibacillus* spp, where pH was altered to such a degree that some populations collapsed (Ratzke, Jonas and Gore, 2018). This demonstrates the drastic effect that changes in pH can have on bacterial populations, through ecosystem engineering by metabolic activity.

Niche-construction is studied in the *P. fluorescens* SBW25 system. When wild-type SBW25 cells are incubated in static liquid microcosms, initial colonists deplete O_2 within the liquid column and an O_2 gradient is established. This environmental modification creates an O_2 rich ecological niche, approximately 200µm deep, at the top of the liquid column (Koza *et al*., 2011). With O_2 now a limiting resource, ecological opportunity is created (Wellborn & Langerhans, 2014) and diversification occurs in which biofilm-forming Wrinkly spreader (WS) mutants dominate. The WS mutant adaptive lineage is better able to exploit the high-O_2 niche created by wild-type SBW25 cells (Schluter, 2000; Yoder *et al*., 2010), and A-L interface biofilm-formation by WS mutants further intensifies the O_2 gradient by blocking O_2 from entering the system (Koza *et al*., 2011; Loudon *et al*, 2016). The clear link between niche-creation and ecological opportunity, and adaptive radiation, suggests the evolution and fitness of the WS mutant may be sensitive to other environmental conditions (Koza *et al*., 2017). *P. fluorescens* SBW25 has a diverse and versatile metabolism, utilising the Enter-Doudoroff pathway in glycolytic metabolism, which suggests growth is energy dependent (Huang and Lin, 2020) and fluorescent pseudomonads are known to produce a range of secondary metabolites (Leisinger and Margraff, 1979). The diverse range of metabolism in *P. fluorescens* SBW25 suggest wild-type cells are capable of acting as ecosystem engineers not just through the uptake of O_2, but also chemically through the production of metabolic waste products and up-take of nutrient. These changes will likely affect growth rates in wild-

type SBW25 populations and effect the evolution and fitness of WS mutants, however this remains un-explored within this system.

Environmental modification through O_2 depletion is well evidence within the *P. fluorescens* SBW25 system (Koza *et al*., 2017). However, chemical and physical differences within the system is evidenced to affect fitness and evolution of the WS mutant. Other physical systems have been used to study WS mutants. By changing the system from microcosm vials to drip-fed columns, the fitness and phenotypic wrinkeality of the WS mutant is significantly altered (Udall, *et al*., 2015). Similarly, altering spatial structure and the physical parameters of the A-L interface within static liquid microcosms alters biofilm-formation, fitness and the evolution of phenotypic diversity of *P. fluorescens* SBW25 populations (Kuśmierska and Spiers, 2016). The composition of nutrients present within the system also has a significant role in the diversification of wild-type SBW25. The diversification of *P. fluorescens* SBW25 has been assessed using different media which changes growth, mutation rate, phenotype (wrinkeality) and biofilm characteristics of WS mutants (MacLaughlin, 2016). Phenotypic diversity also differed when various carbon substrates were utilised as the main nutrient substrate (Venail *et al.*, 2011). *P. fluorescens* SBW25 is typically cultured in KB media which contains a high nutrient level, however when this concentration is decreased, a growth limiting effect is seen (Kassen, Llewellyn and Rainey, 2004; Koza *et al*., 2011; Kuśmierska and Spiers, 2016). These parameters have all been artificially altered within the experiment, however I expect that within this closed system, initial wild-type SBW25 cells have the ability to act as ecosystem engineers and alter the chemical environment. The effect of ecosystem engineering on the nutrient profile of KB* media and how the uptake of nutrients and accumulation of waste metabolites is a knowledge gap within the SBW25 system, however likely affects the diversification and fitness of resulting WS mutants.

Microbes alter the surrounding environment in a number of ways, but most commonly though metabolic products and nutrient up-take (McNally and Brown, 2015). This has been demonstrated in populations of *P. fluorescens* SBW25, where metabolic activity depletes O_2 but less is known about the effects of chemical changes that occur through metabolic activity. However, populations of wild-type SBW25 show lowered fitness when introduced to an environment previously modified by an independently evolved population. This was achieved by competing phenotypes in media that had previously contained growing populations which were removed by filtration (Callahan, Fukami and Fisher, 2014). I conducted similar experiments in Chapter 4 to investigate the decrease in productivity of A-L interface biofilm-forming communities after selection in a serial transfer experiment.

Incubation for three or more days between transfer resulted in a drop in community productivity compared to the initial soil-wash community, which was predicted to be a result of toxic waste product accumulation of and nutrient up-take. Aged media (spent media with prolonged growth period allowing for nutrient depletion, and waste product and metabolite accumulation) was created in a similar manner to Callahan, Fukami and Fisher (2014), and communities re-inoculated into aged media had a significantly drop in community productivity compared to fresh KB*. A comparison with diluted KB* suggested productivity was limited by a combination of toxic metabolites and decrease in nutrient availability. Within this chapter I hope to further investigate the effects of chemical ecosystem engineering within static liquid microcosms using the *P. fluorescens* SBW25 system. I aim to further understand what chemical changes occur through metabolism, and what effect this has on population growth, biofilm-formation and evolutionary processes.

In this chapter the ecosystem engineering by initial wild-type SBW25 colonists will be explored by creating media aged with wild-type SBW25 cells. I hope to demonstrate further chemical changes occur within the environment altering the nutrient profile of KB* microcosms, and these changes have an ecosystem engineering effect by altering the evolution, biofilm characteristics and competitive fitness of WS mutants arising within the population. The increased production of dsDNA and cell biomass within the environment can determine the extent of population growth within the aged media, which will indicate an increase in the amount of metabolic activity within the media. Changes in pH within the environment can indicate changes in chemical composition and alteration in environmental pH is known to cause changes in population growth. Finally, FTIR has been chosen to further determine if chemical changes within media has occurred. Measuring the transmittance of light over a series of wavelengths can indicate changes in molecules present within a liquid. When transmittance is high, there are fewer molecular bonds present to absorb a particular wavelength of light. This provides a simple indication of decreasing nutrients, if transmittance increases at a particular wavelength, or the production of waste products, by a decrease in transmittance. The aged media will then be utilised to reveal the effect of ecosystem engineering on growth, biofilm characteristics and evolution of the WS mutant. I suspect degradation of the environment and accumulation of waste-products will be detected through chemical characterisation, and have a detrimental effect to the process of diversification in wild-type SBW25 populations. I hypothesise a decrease in growth and evolution rate, and a drop in biofilm-characteristics and fitness of both the WS mutants in the aged environments, and in WS mutants evolved within the aged environment.

5.1.1 Chapter research aims

Within the *P. fluorescens* SBW25 system, niche-constructions through the up-take of O_2 results in the diversification and rise of WS mutants, better able to exploit the ecological opportunity created within the system. The aim of this research chapter is to determine the effect of ecosystem engineering through metabolic activity, with the up-take of key nutrients and production of secondary-metabolites and waste products. The effect of ecosystem engineering will be explored by the creation of pre-inhabited media, aged for one - ten days which will be used to explore changes in the diversification *P. fluorescens* SBW25 and the fitness and characteristics of WS mutants.

5.1.2 Research Objectives

1. Create aged media with wild-type SBW25 cells and provide evidence of ecosystem engineering by chemical changes.
    I. Produce media aged for one, three and ten days, and remove all cells to prevent any further metabolic activity.
    II. Conduct chemical characterisation, including changes in pH and FTIR spectroscopy to demonstrate chemical change.
2. Explore the effect of the modified environment on the evolution of the WS mutant.
    I. Evolve WS mutants from wild-type SBW25 within each of the aged media to determine impact on evolution rate.
    II. Compare biofilm characteristics and fitness of WS mutants from different aged media.
    III. Compare WS mutant fitness and characteristics in the aged environment in which it evolved in and standard KB* to determine if adaption to the engineered environment has occurred.
3. Determine any inhibitory effects of aged media on wild-type SBW25 and WS mutant populations
    I. Compare growth of wild-type SBW25 in aged media and standard KB* to determine if growth is inhibited.
    II. Compare biofilm characteristics of WS mutants in aged media and standard KB* to determine if chemical changes affect biofilm-formation.

III. Carry out pair-wise fitness assays in aged media in static and shaken conditions to determine if competitive fitness is changed by aged media.

Due to the difficulty in evolving and retrieving WS mutants from the three and ten - day old aged media, and the closure of all campus facilities between March 2020 and August 2020 during the COVID-19 pandemic Research Aim 2 could not be fully completed. In particular, some initial un-replicate experiments could not be further developed. The biofilm characterisation and fitness comparison (Objective 2. III) was still carried out between WS mutants evolved in one-day old media and standard WS mutants, but could not be carried out for the three and ten-day aged media. Nonetheless, I have decided to present all findings in this chapter with the appropriate caveats and suggested future work.

5.2 Results

5.2.1 Ecosystem engineering by *P. fluorescens* SBW25 causes chemical changes in media

Environmental modification through the uptake of resources and production of waste production can alter the chemical and physical environment. Within the *P. fluorescens* SBW25 system ecosystem engineering through the depletion of O_2 in the liquid column, creating an O_2 gradient within the environment is well evidenced. This modifies the environment creating a high-O_2 ecological niche at the top of the liquid column, and only lineages suitably adapted to colonisation at the A-L interface can exploit this ecological opportunity. *P. fluorescens* SBW25 has diverse metabolism, so the resulting effect of nutrient uptake and waste production is likely to modify the chemical environment. Prior to testing the effects of ecosystem engineering, aged media was created and chemical analysis was carried out to evidence chemical changes. It was expected that by testing pH, FTIR spectrometry and dsDNA quantification, changes between fresh KB* and aged media would be detected with the greatest changes seen in media with a longer aging period. Similarly, a change in the biomass recovered from each aging population was expected, with an increase in recovered biomass recovered as the age of the media increased, indicating increased metabolism.

Large quantities of aged media were created under shaken conditions to maximise metabolic uptake and toxin production by inoculating KB* media wild-type SBW25 cells, and incubated for one, three and ten days. After biomass was removed by centrifugation the media was used to create 6ml microcosms, creating modified microcosms which were autoclaved to prevent any further growth. KB* microcosms were also double-autoclaved (KB*-DA) to provide appropriate control. Autoclaving aged media may result in the loss of some heat sensitive toxins, however this was needed to ensure no further cell growth occurred. Aliquots from each aged media were used for chemical analysis comparing pH, recovered biomass, dsDNA quantification and FTIR spectroscopy. Significant chemical differences were observed between the aged media and standard KB*, and between each of the aged media (Figure 5.1, TK-HSD, $\alpha = 0.05$). No significant differences were seen between KB* and KB* microcosms after double autoclaving, confirming a second sterilisation process had no chemical effect to KB* media. As media aged the pH increased from approximately pH 6.8 (KB*, KB*-DA and one-day old media) to pH 7.01 and pH 7.37 for three-day old and ten-day old media respectively. The biomass recovered also increased as media aged, reaching plateau at three days of aging (6037.3 ± 888 g), after which no significant increase was seen in ten-day old media. The quantity of dsDNA (ng/µl) was significantly higher in one- day old media (463 ± 5.2 ng/µl), compared to three and ten-day

old media (418.7 ± 5.2 and 415.9 ± 5.2 ng/µl) which still contained a significantly high level of dsDNA compared to standard KB* where no dsDNA was detected.

Chemical changes in aged media were further explored with principal component analysis, which illustrated changes, PC 1 (x-axis) 81.035 %; PC 2 (y-axis), 15.05 % (PC 3 is not significant based on the Eigen values), showing a shift towards dsDNA in one-day media, recovered biomass in three-day media, and pH in ten-day old media (Figure 5.2).

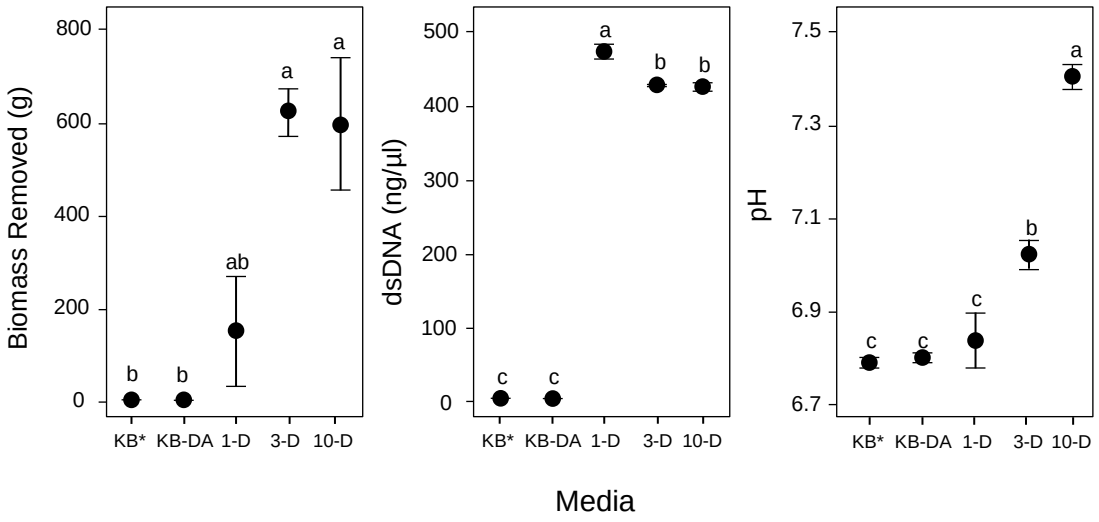


Figure 5.1. Chemical changes in media aged with *Pseudomonas fluorescens* SBW25 wild-type compared to standard KB* media. Media aged with populations of wild-type cells were compared prior to autoclaving, measuring changes in biomass recovered (g), dsDNA (ng / µl) and pH. Means ± SE are shown (n = 2) and means not linked by the same letter are significantly different (TK-HSD, α = 0.05).

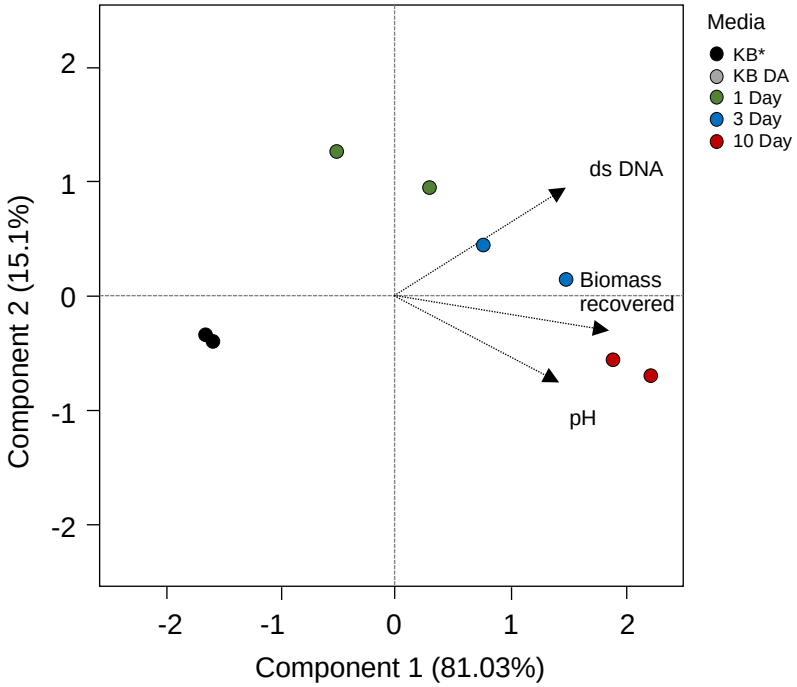


Figure 5.2. Principal component analysis comparing aged media, standard KB* and KB*-DA using biomass recovered (g), dsDNA (ng / µl) and pH data. PC 1 (x-axis), 81.03 %; PC 2 (y-axis), 15.1 % (PC 3 is not significant based on the Eigen values). All aged media show changes in chemical space compared to standard KB* media.

Fourier-transform infrared spectroscopy also demonstrated changes in aged media. The transmittance (%) of each aged media and KB* was measured between the wavenumbers of 400.16 cm^{-1} to 4000.12 cm^{-1} and were compared using hierarchical cluster analysis (HCA). HCA could differentiate each of the aged media, and the KB* and KB-DA (Figure 5.3). A small section of the FTIR spectra was chosen at random (wavenumbers 2909 cm^{-1} to 2939 cm^{-1}) and shows a decrease in transmittance in three and ten-day old media, with very small changes in seen in the one-day media compared to KB* (Figure 5.3). The transmittance of KB* and KB-DA could not be differentiated at these wavenumbers.

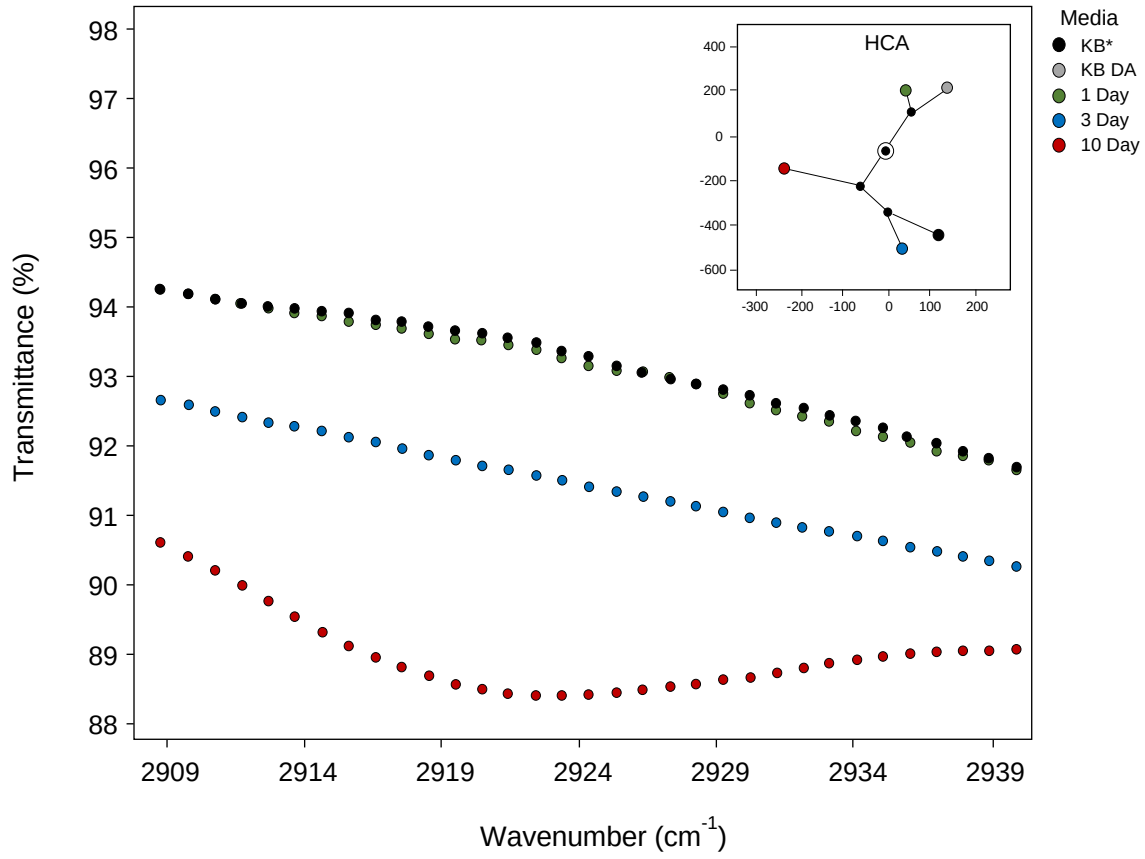


Figure 5.3. Section of FTIR spectra and HCA of FTIR spectra of aged media, standard KB* and KB*-DA. FTIR spectra from wavenumbers 2909 cm^{-1} to 2939 cm^{-1} show changes in transmittance (%) in aged media compared to standard and double autoclaved KB*. HCA (top right graph) demonstrates significant difference in the full FTIR spectra from wavenumbers 400.16 cm^{-1} to 4000.12 cm^{-1} between aged media.

5.2.2 *P. fluorescens* SBW25 growth and evolution rate decreases in aged media

Environmental modification through ecosystem engineering and niche creation is known to effect evolutionary processes. WS mutants are evidenced to arise after three days of static incubation, when wild-type SBW25 static populations become dominated by WS mutants. It was expected that chemical changes will affect the rate in which WS mutants arise within wild-type SBW25 populations, and the characteristics of these mutants. WS mutants evolved within different media are known to have altered biofilms characteristics, so mutants evolved in an environment chemically modified by initial colonists are also expected to have altered characteristics. First the percentage of WS mutants arising in wild-type SBW25 populations over a ten day evolution experiment was calculated, where the total number of WS mutants

and wild-type SBW25 colonists were counted over three populations which were plated after one, three, five and ten days. Evolution experiments were then carried out in each of the aged media created (one, three and ten-day old), with three days static incubation, to assess changes in evolution rates as a result of environmental modification.

Within standard KB* microcosm the percentage of WS mutants arising in wild-type SBW25 populations was exponential, with the percentage of WS remaining low after one and three days (0 and 0.18 %), rising to 2.4 % after five days of static incubation and finally 28.6 % of the population consisted of WS mutants after ten days of incubation (Figure 5.4).

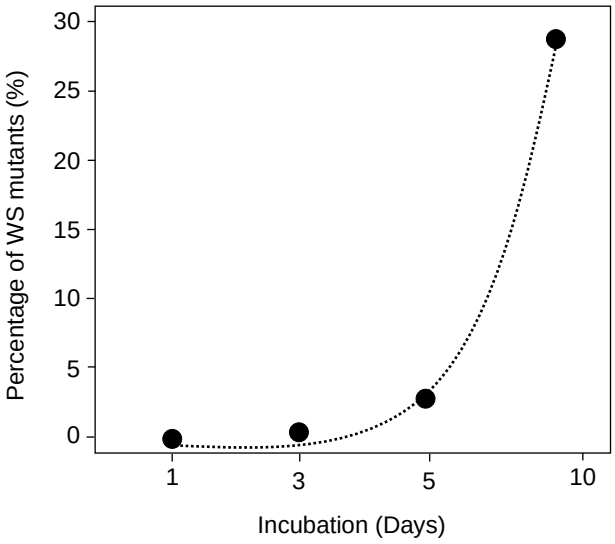


Figure 5.4 The percentage of WS mutant arising in standard static KB* microcosms inoculated with wild-type SBW25. Upon static incubation of wild-type SBW25, WS mutants start to arise in the population after three days. The total percentage of WS mutants across three serial dilutions are shown over a ten-day period.

The percentage of WS mutants arising in wild-type SBW25 populations when incubated in modified microcosms with aged media decreased compared to fresh KB* microcosms. Within standard KB* microcosms WS mutants made up 0.18% of the population after three days of incubation. This decreased to 0.16% in one-day old media, 0.13% in three-day old media, and of the replicate populations plated from populations in the ten-day old media, no WS mutants were observed. The CFU's of wild-type SBW25 was also notably smaller in the three and ten-day aged media, suggesting aged media also had an inhibiting effect in wild-type SBW25 cells. With replicate populations (n = 3), a static growth assay was carried out to determine if aged media was inhibiting wild-type SBW25 growth, and CFU's were counted

after three days of static incubation. CFU were compared on plates diluted to $x10^{-7}$. Within standard KB* microcosms wild-type SBW25 had 37.6 ± 1.8 $x10^{-7}$ CFU. This significantly changed in aged media (Figure 5.5), with a decrease in CFU as the age of the media increased (one-day 23.3 ± 6.06, three-day 9.6 ± 2.02, ten-day 7.0 ± 3.4 $x10^{-7}$ CFU, TK-HSD, α = 0.05). This suggests media aged by the wild-type SBW25 is not just inhibiting to WS mutant cells, but also to the engineering wild-type SBW25 strain.

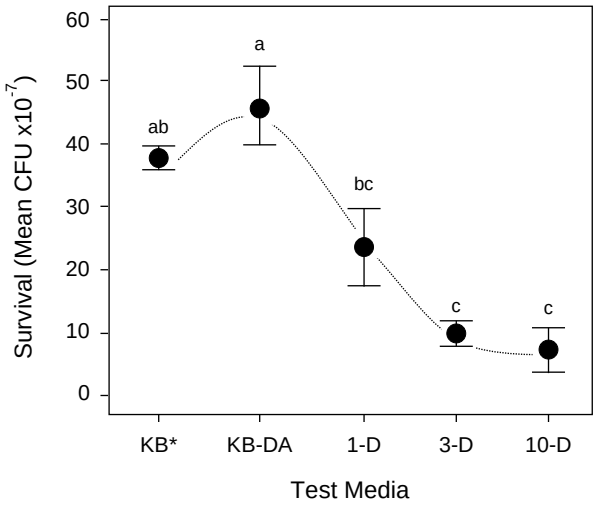


Figure 5.5 Wild-type SBW25 survival significantly decreases in aged media. The chemical changes caused by wild-type SBW25 metabolism and nutrient up-take causes a toxic effect to the population. The survival of wild-type SBW25 significantly drops as the age of the media increases, shown by a decrease in the number of CFU at $x10^{-7}$ in static cultures after three days of static incubation compared to standard KB*. Means ± SE are shown, means not linked by the same letter are significantly different (TK-HSD, α = 0.05).

From the evolution experiments in aged media any present WS mutants were collected and re-streaked for further experimentation. Three WS mutant colonies from the one-day old media were successful re-streaked and stored at - 80˚C. This was attempted for the three-day old media WS mutant colonies, however the WS recovered were unstable and reverted back to wild-type SBW25 colonies once re-streaked, and no WS mutants were observed in the ten-day evolution experiment.

5.2.3 Changes in biofilm characteristics, fitness and adaption of WS mutants evolved in one-day aged media.

It is known that altering the parameters of the microcosm system and the composition of media alters the phenotype, evolution and biofilms characteristics of the WS mutant (Udall, *et al*., 2015; Kuśmierska and Spiers, 2016, Venail *et al*., 2011; MacLaughlin, 2016). This would suggest that chemical changes caused by wild-type SBW25 may also cause significant changes in characteristics of WS mutants evolved within an engineered environment. However, mutants arising within a population are likely adapted to the surrounding environment, suggesting WS mutants evolved in one-day media could perform better in this environment compared to the archetypal WS mutant. To determine if the diversification of *P. fluorescens* SBW25 in one-day aged media causes changes in the characteristics of evolved WS mutants the morphology and biofilm characteristics of the recovered WS mutants from the evolution experiments were compared in standard KB* media. Three WS mutants were recovered from the one- day aged media (hereafter aged media WS mutants, AM-WS-1, AM-WS-2 and AM-WS-3). Adaption of the AM-WS mutants were explored by comparing biofilm characteristics and fitness to three archetypal WS mutants selected after three days of static incubation in KB*, in both standard KB* media and one-day aged media. This would indicate if WS mutants evolved in one-day media have adapted to the aged environment, which would result in higher fitness and biofilm characteristics in the AM–WS mutants compared to the archetypal WS mutants. I expect that AM-WS mutants will show a decrease in biofilm characteristics and altered colony morphology compared to the archetypal WS mutants when compared in KB* as a result of evolving with an environment depleted in nutrients and containing waste products. However, I suspect that the AM-WS mutants will be adapted to growing in the one-day aged media, therefore will outperform the archetypal WS mutants in both competitive fitness and biofilm characteristics when compared in the one-day aged media.

Three WS were collected from three-day evolution experiments from KB* media (archetypal WS) and one-day aged media (AM-WS). Colony morphology of the archytpal and AM-WS were compared on KB* plates after recovery from evolution experiments. All three AM-WS mutants recovered from aged media were visually smaller on agar plates compared to archetypal WS mutants (Figure 5.6), across all replicate dilution plates (10^{-3} to 10^{-8}), confirming changes in colony morphology as a result of evolution within an aged environment. The biofilm characteristics of the AM-WS mutants and archetypal WS mutants were significantly different when compared in KB* microcosms (Figure 5.7). All AM-WS

mutants had a significant decrease in growth compared to two of the three WS mutants (AM-WS mutants 1.39 ± 0.05, 1.56 ± 0.06 and 1.47 ± 0.02 OD_{600}, WS mutants 1.80 ± 0.40 and 1.84 ± 0.45 OD_{600}, TK-HSD, α = 0.05). There were significant differences in attachment, with all AM-WS mutants showing significantly lower attachment to WS mutants (AM-WS mutants 0.33 ± 0.01, 0.30 ± 0.03 and 0.31 ± 0.02 mean A_{570}, WS mutants 0.45 ± 0.03, 0.40 ± 0.04 and 0.45 ± 0.4 $A_{570,}$ TK-HSD, α = 0.05). Finally, the AM-WS mutants had significantly lower strength than two of the three archetypal WS mutants (AM-WS mutants 0.023 ± 0.007, 0.012 ± 0.001 and 0.015 ± 0.00 grams, WS mutants 0.07 ± 0.02 and 0.1 ± 0.02 grams, K-W Wilcoxon, α = 0.05).

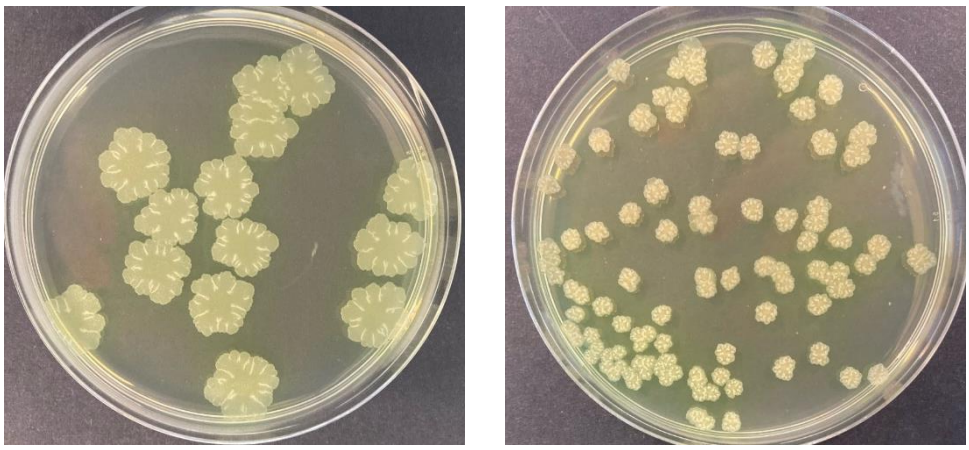


Figure 5.6. The morphology of WS mutants evolved in one-day aged media are visually smaller. The morphology of WS mutants evolved in one-day aged media were compared to archetypal WS mutants on KB* plates. The WS mutants recovered from the one-day aged media were visually smaller than WS mutants evolved in standard KB* media. This image is descriptive only.

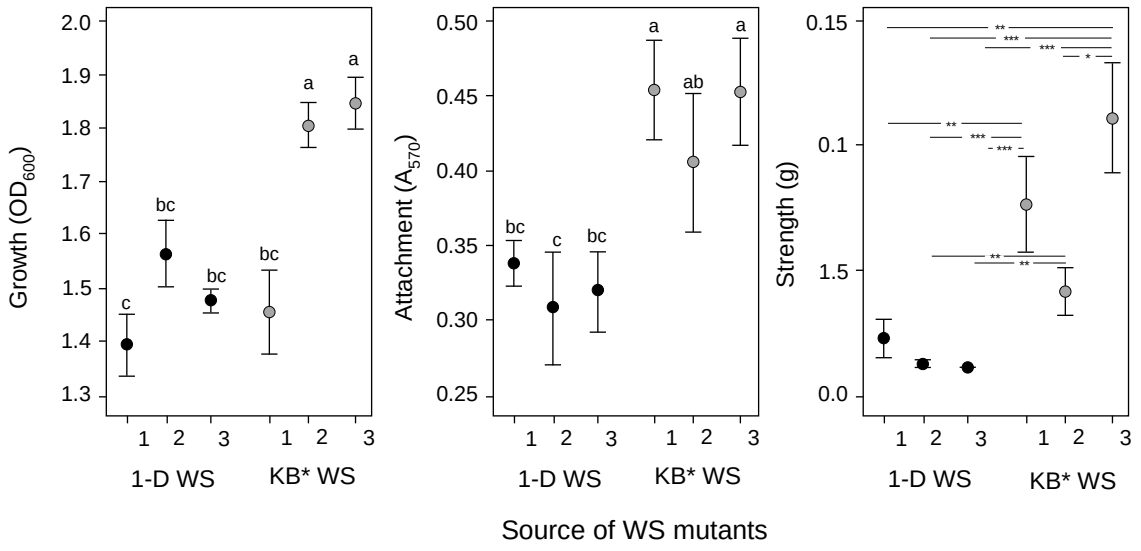


Figure 5.7. The biofilm characteristics of WS mutants evolved in one-day media are significantly different to WS mutants evolved in standard KB*. Total growth (OD_{600}), biofilm attachment (A_{570}) and **b**iofilm strength (g) of WS mutant populations evolved in one-day aged media are significantly lower than WS evolved from standard KB*. Means ± SE are shown (n = 8), means not linked by the same letter are significantly different (TK-HSD, α = 0.05). Significant differences in strength are indicated by asterisks (Wilcoxon method; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

To determine if AM-WS mutants are adapted to the media in which diversification occurred, the combined biofilm assay was repeated, comparing biofilm characteristics of the AM-WS mutants and WS mutants in both standard KB* and one-day age media. Significant differences were observed between both the media and the WS mutant strains (Figure 5.8), and this media and strain effect was confirmed using a modelling approach (GLM; strain effect $P = 0.000$; media effect $P = 0.000$; replicate effect $P = 0.74$).

Growth (OD_{600}) of the archetypal WS mutant and AM-WS mutants were significantly lower in one-day aged media compared to standard KB* media, but no significant differences were observed between the WS mutants (KB* media; WS mutant 1.87 ± 0.01 and AM-WS mutants 1.91 ± 0.04, 1.87 ± 0.03 and 1.93 ± 0.02 $OD_{600.}$ One-day aged media; WS mutant 1.52 ± 0.05 and AM-WS mutants 1.39 ± 0.05, 1.39 ± 0.06 and 1.41 ± 0.06 OD_{600}, T-K HSD, α = 0.05). Attachment of the WS mutant was significantly higher in both KB* and one-day aged media compared to all three AM-WS mutants, and the WS mutant showed significantly higher attachment in one-day media compared to KB* (archetypal WS mutant attachment in KB* 0.46 ± 0.06 and one-day media 1.53 ± 0.05 $A_{570,}$ T-K HSD, α = 0.05). The AM-WS mutants also demonstrated higher attachment in the one-day aged media compared to KB*

media (one-day aged media; 0.56 ± 0.02, 0.65 ± 0.02 and 0.56 ± 0.05 and KB* media; 0.28 ± 0.02, 0.20 ± 0.01 and 0.19 ± 0.01 $A_{570,}$ T-K HSD, α = 0.05). Finally, biofilm strength (shown here as the Log of grams (g) so a GLM model and PCA could be made with the CBA data as original strength data was not normally distributed) of the AM-WS mutants were not significantly different in two of the three mutants when compared in the two media types (AM-WS-1 KB* -1.64 ± 0.11 and one-day media -1.82 ± 0.05; AM-WS-2 KB* -1.60 ± 0.09 and one-day media -1.86 ± 0.04, Log grams, T-K HSD, α = 0.05). The third AM-WS mutant was significantly higher in KB* compared to one-day aged media (AM-WS-3 KB* -0.76 ± 0.11 and one-day media -1.86 ± 0.04, Log grams, T-K HSD, α = 0.05). Biofilm strength of the archetypal WS mutant was not significantly different between media types, but was significantly higher compared to AM-WS mutants in both media types (WS mutant KB* -0.52 ± 0.01 and one-day media -0.76 ± 0.11, Log g, T-K HSD, α = 0.05).

The differences in biofilm characteristics between the AM-WS mutants and the archetypal WS mutant in both KB* and one-day aged media was further confirmed with principal component analysis (Figure 5.9); PC 1 (x-axis) 81.035 %; PC 2 (y-axis), 15.05 % (PC 3 is not significant based on the Eigen values). The PCA shows the archetypal WS mutant mean data in KB* media is directed towards increased growth and strength, and in the one-day aged media directed towards attachment. The AM-WS mutants are clustered away from strength and growth in one-day media, and a clustered towards growth in the KB* media.

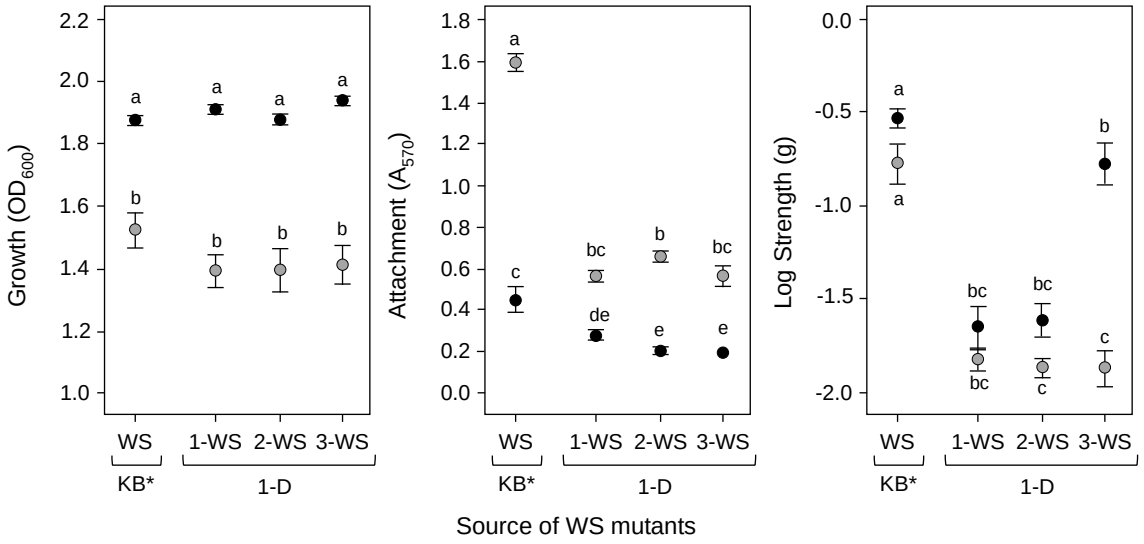


Figure 5.8. Changes in biofilm characteristics between the archetypal WS mutants and WS mutants evolved in one-day aged media in both KB* and one-day aged media Biofilm strength (Log grams), attachment (A_{570}) and total growth (OD_{600}) after three days of static incubation were compared between the AM-WS mutants (WS mutants evolved in one-day aged media) and the

archetypal WS mutant in both standard KB* media (black circles) and one-day aged media (grey circles). Total growth (OD_{600}) and biofilm strength (Log g) were significantly lower in the AM-WS mutants when incubated in the one-day aged media compared to KB*, however biofilm attachment (A_{570}) was significantly higher. The archetypal WS mutant had significantly higher attachment and strength compared to the AM-WS mutants across both media types, however no significant differences were seen in growth. Means ± SE are shown (n = 8), means not linked by the same letter are significantly different (TK-HSD, α = 0.05).

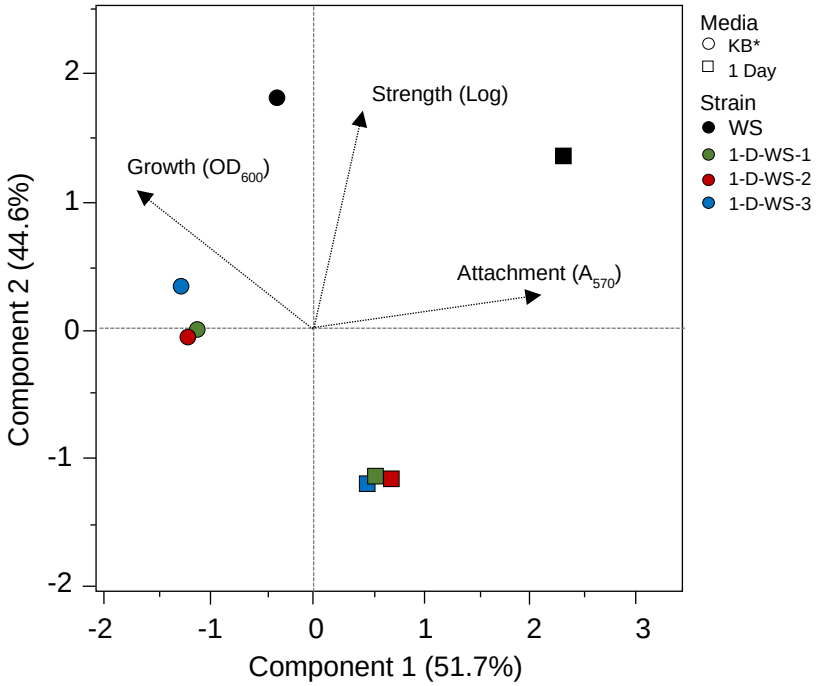


Figure 5.9. Principal component analysis comparing biofilm characteristics of the archetypal WS and AM-WS mutants in standard KB* and one-day aged media. PC 1 (x-axis), 51.74 %; PC 2 (y-axis), 44.6 % (PC 3 is not significant based on the Eigen values). The archetypal WS mutant and 1 - day WS mutants show changes in KB* in biofilm-associated phenotypic space when compared on KB* and one-day aged media.

Competitive fitness (W) of a strain can be utilised to show adaption within an environment, where an increase in competitive fitness would suggest adaption. It was therefore expected the AM-WS mutants would show increased fitness in the one-day aged media compared to the archetypal WS mutant. Pairwise fitness assays were carried out at a 1:1 ratio (Figure 5.10), comparing the competitive fitness of the AM-WS mutants and archetypal WS mutant against *P. fluorescens* SG70 (Gehrig, 2005). As the wild-type SBW25 strain can form a VM biofilm under standard conditions at Abertay University, dipyridyl-tiron is added to prevent biofilm-formation occurring (Koza *et al.,* 2009). However, this may also affect the WS mutant

fitness so it was decided to use SG70, the wild-type SBW25 with the wss operon deleted, within this chapter. No significant differences in competitive fitness was found in the archetypal WS mutant when compared in both KB* and one-day aged media (KB* W = 1.25 ± 0.10 and one-day media W = 1.01 ± 0.07, T-K HSD, α = 0.05) with the WS mutant retaining a small competitive fitness over SG70. The competitive fitness of the AM-WS mutants were significantly higher than the archetypal WS mutant in both media types, and two of the three the AM-WS mutants had a significant increase in competitive fitness in KB* media compared to one-day aged media (AM-WS-1 KB* W = 6.21 ± 0.17 and one-day media W = 2.22 ± 0.39; AM-WS-2 KB* W = 6.02 ± 0.47 and one-day media W = 2.62 ± 0.14; AM-WS-3 KB* W = 2.09 ± 0.20 and one-day media W = 2.43 ± 0.02, T-K HSD, α = 0.05). This data confirms changes in competitive fitness between the AM-WS mutants and the archetypal WS mutant, with AM-WS mutants gaining a significant competitive fitness increase compared to the WS mutant in both media types.

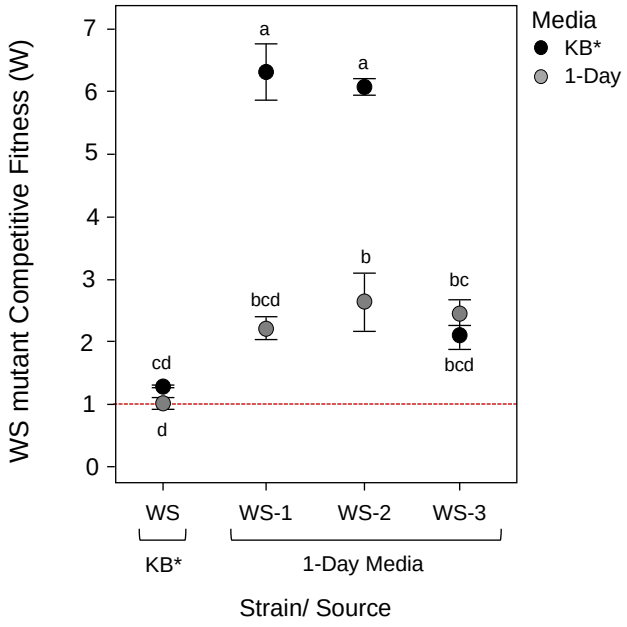


Figure 5.10. The competitive fitness of WS mutants evolved in one-day media are significantly higher that the archetypal WS in both KB* and one-day aged media. Pairwise fitness assays were carried out with initial cell ratios of 1:1 (or close to) and plated after 72 h of static incubation. The fitness (W) of the three AM-WS mutants and the archetypal WS mutant compared to *P. fluorescens* SG70 is shown in both KB* (black circles) and one-day aged media (grey circles). The red dotted line at 1 indicates a fitness of 1, where the fitness of each strain would be equal. A fitness of W<1 indicates that the strain is at a disadvantage. A fitness of W> 1 indicates the strain has the competitive advantage. Means ± SE are shown (n = 5), means not connected by the same letter within the same panel and statistically different (T-K HSD, α = 0.05).

5.2.4 Archetypal WS mutant biofilm characteristics and fitness are significantly altered in aged media

The effect of an altered chemical and physical environment does not just affect evolutionary process, but also effects the fitness and characteristics of residing species. Changes in the abiotic environment such as pH, temperature or redox potential can create variation in key microbial processes such as metabolism and growth. As WS mutants evolved in the one-day old media had a decrease in biofilm characteristics, it was expected that the archetypal WS mutants would produce weaker biofilms in each of the aged media, with lower total growth. If so, this would suggest the WS competitive fitness would also change in the aged environment. The biofilm characteristics and growth of the archetypal WS mutant was compared in the aged environments, and competitive fitness compared to wild-type SBW25 was investigated under static and shaken conditions.

As the age of the media increased, the total growth of WS mutant static populations decreased. All combined biofilm assay measurements showed significant changes in aged media (Figure 5.11). A significant decrease in growth was seen between the aged media and standard KB*, with a larger decrease seen as the media age increased (KB* 1.8 ± 0.03, one-day 1.7 ± 0.03, three-day 1.18 ± 0.03 and ten-day 1.06 ± 0.05 $OD_{600,}$ TK-HSD, α = 0.05). Similarly, strength (g) also showed a significant decrease in aged media, with no significant differences seen in the one-day aged media compared to standard KB* (KB* 0.3 ± 0.009 and one-day 0.41 ± 0.05 g), a small but not significant difference in three-day media, and a significant decrease found in ten-day media (three-day 0.22 ± 0.05 and ten-day 0.04 ± 0.01 g, TK-HSD, α = 0.05). Small changes in attachment were observed, with a small but not significant increase within the one-day compared to KB* (KB* 0.30 ± 0.02 and one-day 0.38 ± 0.01 A_{570}), a small but not significant decrease in three-day media and a significant decrease found in the ten-day media (three-day 0.27 ± 0.01 and ten-day 0.19 ± 0.01 $A_{570,}$ TK-HSD, α = 0.05). Changes in biofilm characteristics in WS mutants in aged media was confirmed with principal component analysis, PC 1 (x-axis) 67.6 %; PC 2 (y-axis) 26.4 % (PC 3 is not significant based on the Eigen values), showing a shift towards attachment in one-day media compared to standard KB*, and the three and ten-day media shifting away from strength and growth compared to standard KB* (Figure 5.12).

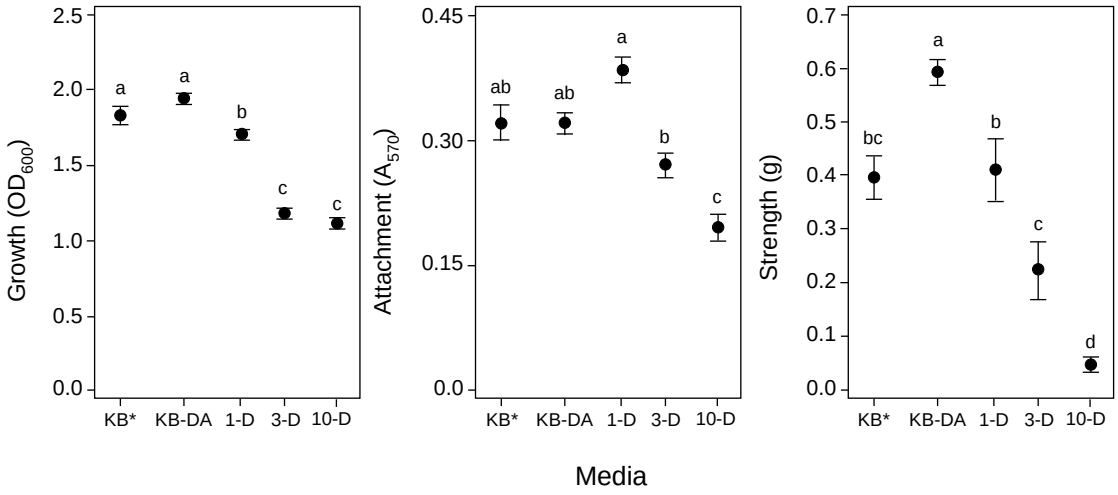


Figure 5.11. The biofilm characteristics of WS mutants change in aged media. Biofilm strength (g), attachment (A_{570}) and total growth (OD_{600}) of WS mutant populations after three days of static incubation in each of the aged media (one, three and ten-days) and standard KB*. Means ± SE are shown (n = 8), means not linked by the same letter are significantly different (TK-HSD, α = 0.05).

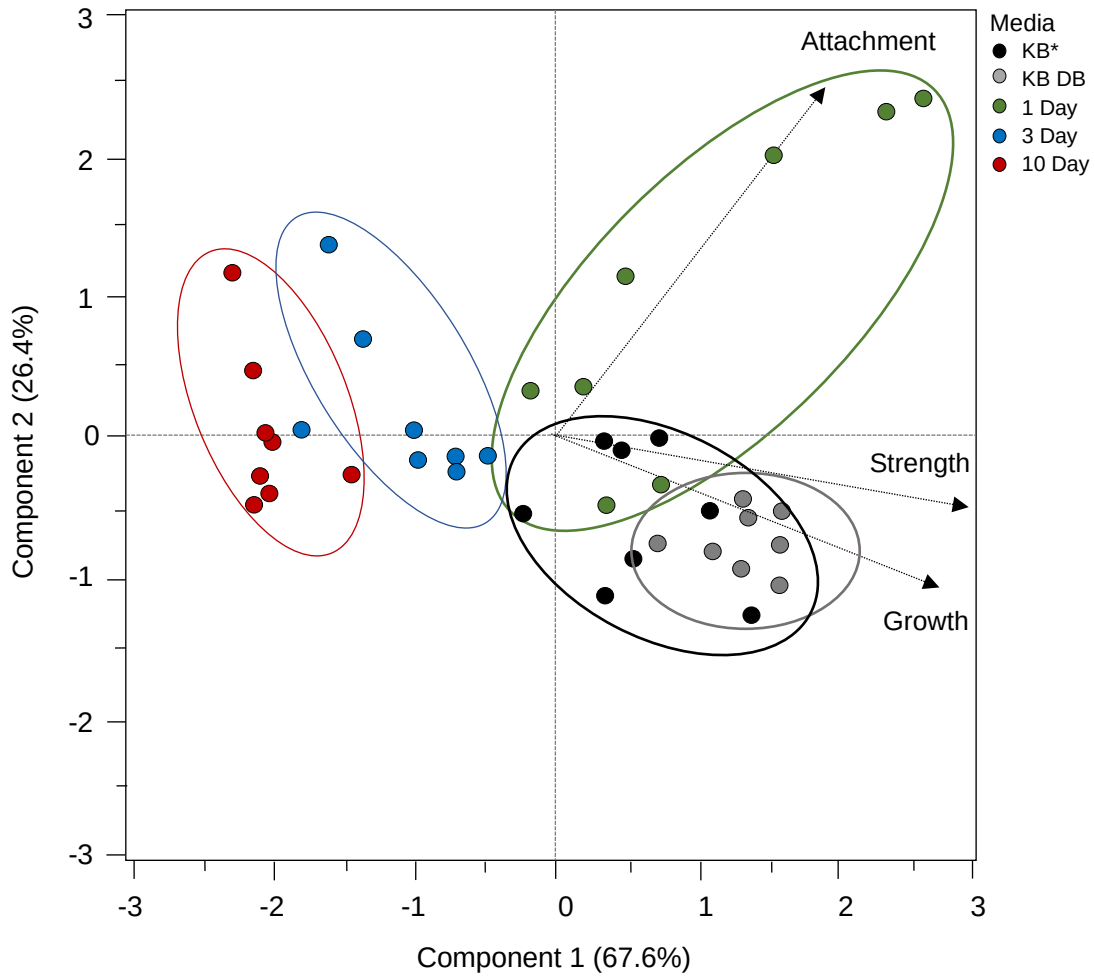


Figure 5.12. Principal component analysis comparing biofilm characteristics of the WS mutant in standard KB* and aged media. PC 1 (x-axis) 67.6 %; PC 2 (y-axis) 26.4 % (PC 3 is not significant based on the Eigen values). All aged media show changes in chemical space compared to standard KB* media.

The WS mutant has a known competitive fitness over wild-type SBW25 in static liquid microcosms due to increased access to O_2 through biofilm-formation. This fitness advantage is lost in a shaken environment where biofilm-formation is not possible, and the production of cellulose by WS mutants is wasted. This confirms that a competitive fitness can be specific to a given environment, and any changes within that environment can results in the loss of fitness. This was already demonstrated within the *P. fluorescens* SBW25 system, where the removal of the O_2 gradient by shaking incubation where O_2 is constantly introduced throughout the culture, results in the loss of the WS mutant competitive fitness. Chemical ecosystem engineering from the metabolic activity of wild-type SBW25 has now been evidenced within this system, and this results in altered growth rates in wild-type SBW25 and significant changes in the evolution and biofilm characteristics of WS mutants. This indicates that the environmental conditions have now significantly changed, suggesting the WS

mutant competitive fitness could also be altered. I suspected that in shaken cultures the WS mutant will continue to be at a disadvantage, as not only is biofilm-formation not possible, but cells are also subjected to toxic metabolites and limited nutrients limiting population growth. In static conditions, the same toxic metabolites are present, and biofilm characteristics of the WS mutant biofilm have been shown to be weaker, suggesting this could also decrease the fitness advantage of the WS mutant.

Pair-wise fitness assays were carried out in both static and shaken conditions in standard KB* and the aged media. Fitness assays were carried out using the WS mutant and the *P. fluorescens* strain SG70 (Gehrig, 2005). In static liquid microcosms containing aged media the WS mutant retained a fitness advantage over wild-type SBW25 (Figure 5.13), with a small increase in competitive fitness in one-day media, and a significant increase in fitness in the ten-day media compared to standard KB* (KB* W = 1.15 ± 0.09, one-day W = 1.38 ± 0.16 and ten-day W = 1.87 ± 0.04, TK-HSD, α = 0.05). In shaken conditions, where the WS mutant does not have a competitive advantage in standard KB*, the fitness of the WS mutants significantly dropped as the media age increased, with significant differences in the three-day and ten-day media compared to KB* (KB* W = 1.0 ± 0.06, three-day W = 0.73 ± 0.05 and ten-day W = 10.6 ± 0.06, TK-HSD, α = 0.05).

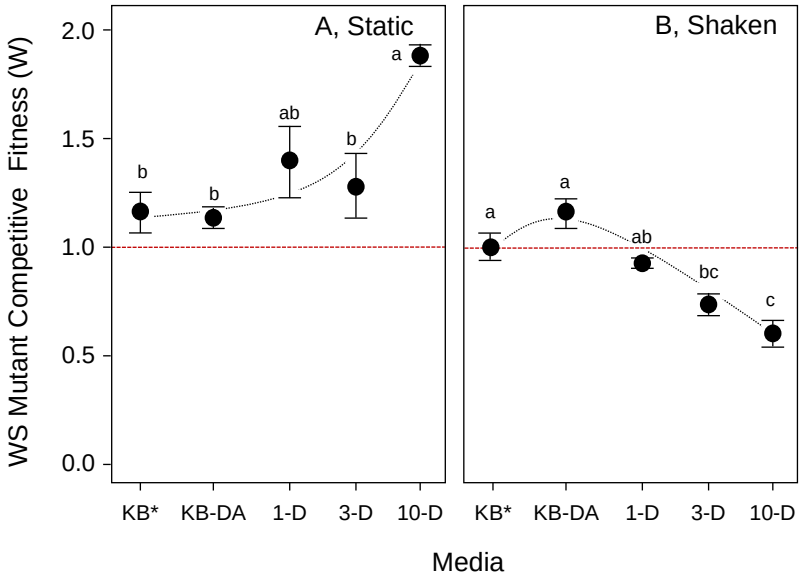


Figure 5.13. Pairwise competitive fitness between wild-type SBW25 and WS mutant in KB* and aged media. Pairwise fitness assays were carried out with initial cell ratios of 1:1 (or close to) and cultures were incubated statically (A) or with shaking (B) for 72 h before serial dilution and plating. The red dotted line at 1 indicates a fitness of 1, where the fitness of each strain would be equal. A fitness of W<1 indicates that the strain is at a disadvantage. A fitness of W> 1 indicates the strain has

the competitive advantage. Means ± SE are shown (n = 5), means not connected by the same letter within the same panel and statistically different (T-K HSD, α = 0.05). Trend lines (dashed curves) are descriptive only.

These results conclude that chemical changes within the media caused by nutrient up-take and waste product accumulation does not affect the fitness of the WS mutant in static liquid microcosms, and in the most extreme conditions where media has been aged for ten days, the WS mutants fitness actually increases. The converse is found in shaken conditions, as the age of the media increases the WS fitness significantly decreases compared to wild-type SBW25. A negative correlation in shown between static and shaken incubation as the age of the media increases (ρ = -0.9, P – 0.03, Figure 5.13).

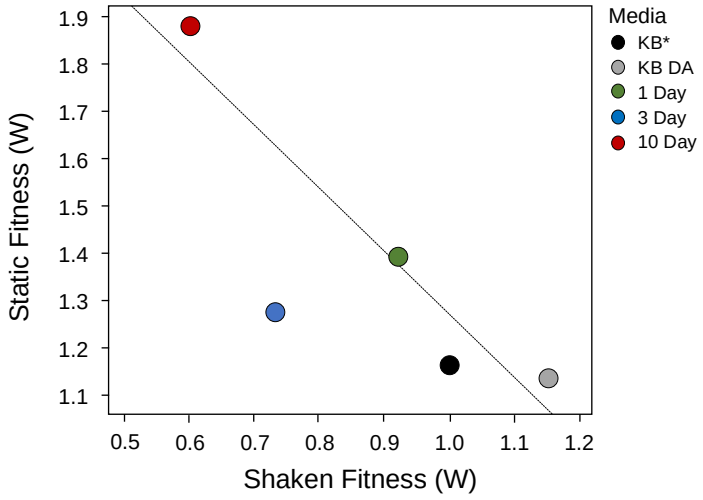


Figure 5.14. Correlation between shaken and static incubation of WS mutant fitness in aged media. Spearman's correlation coefficient (ρ) shows a negative correlation between the static and shaken incubation as the age of the media increases (ρ = -0.9, P – 0.03).

5.3 Discussion

Within the *P. fluorescens* SBW25 system, initial wild-type colonists act as ecosystem engineers through the depletion of O_2 in the lower liquid column, creating a high-O_2 ecological niche at the top of the liquid column. WS mutants arising within the population are better able to exploit the ecological opportunity created by environment modification, and through biofilm-formation at the A-L interface can benefit from increase access to O_2. O_2 is a well evidenced limiting resource within the *P. fluorescens* SBW25, however nutrients are also a limiting resource, however it is less known at what point nutrients and chemical alteration becomes limiting with the system. Bacteria act as ecosystem engineers thorough simple processes such as metabolism, releasing waste products into the environment and depleting available nutrients ((McNally and Brown, 2015). This would therefore affect the growth of future lineages. This has been evidence in static liquid microcosms in Chapter 4, where a prolonged incubation period of three to six days resulted in a decrease in community productivity. Further investigation suggested a combination of nutrient-uptake and toxic metabolite accumulation had a growth limiting effect on the community. This was demonstrated by creating aged media, and re-introducing community samples into the aged environment, which resulted in lower growth compared to fresh KB* microcosms. Similarly, diluted nutrients also had a similar effect, suggesting the decrease in community productivity found in the community serial transfer experiments was a result on nutrient depletion and possible toxic waste product accumulation.

Within this chapter I intended to further explore the effect of chemical ecosystem engineering in static liquid microcosms using the *P. fluorescens* SBW25 system. In populations of *P. fluorescens* SBW25 the chemical composition and concentration of nutrients within the liquid media is known to effect growth rates, phenotype and fitness of WS mutants arsing within the population (Kassen, Llewellyn and Rainey, 2004; Venail *et al.,* 2011; MacLaughlin, 2016), however in these cases media has been changed or diluted artificially by the experimenter. Wild-type SBW25 cells are likely to change the chemical composition of the media through metabolism. However, the extent and effect of chemical changes within the media through wild-type SBW25 metabolism remains unexplored. This chapter looked to create media aged by wild-type SBW25 cells to determine if chemical changes were detectable to provide evidence of ecosystem engineering. The effect of these chemical changes were then tested with populations of wild-type SBW25 and WS mutants to determine if an aged environment had any significant impact on growth, biofilm characteristics, and the evolution and fitness of the WS mutant. How organism impact the surrounding environment can have a profound effect on residing organisms and exploring

ecosystem engineering within a system can provide an insight to the ecological dynamics within.

5.3.1 Chemical changes are detected in media aged by wild-type SBW25 cells

Bacteria metabolism is a chemical process involving the uptake of key nutrients and substrates to generate energy, which results in the production of a range of metabolic waste products or secondary metabolites. In addition, cells often produce scavenging molecules to enhance nutrient-up, resulting in further release of new molecules into the surrounding environment. This will result in chemical changes within the environment, typical of ecosystem engineering. Within the *P. fluorescens* SBW25 system, wild-type SBW25 is already evidenced to act as ecosystem engineer through the uptake of O_2, depleting O_2 in the lower liquid regions, creating a high-O_2 ecological niche at the top of the liquid column (Koza *et al*., 2017). It is also expected that initial wild-type SBW25 colonist will act as chemical ecosystem engineering through the up-take of nutrients, production of siderophores for iron scavenging and the production of waste products and secondary metabolites. To investigate ecosystem engineering within the *P. fluorescens* SBW25 system, KB* media was aged with wild-type SBW25 cells for one, three and ten days. Once cells were removed, this media could then be analysed for chemical changes, and be used to create modified microcosms to test the effect of a chemically engineered environment.

It was expected that growing populations of wild-type SBW25 would chemically alter KB* media, with an increase in biomass and dsDNA recovered from the media as the media aged, a decrease in media pH caused by the release of acidic waste products and H+ ions, and a change in FTIR spectrum transmittance as a result of the up-take of KB* nutrients and production of waste products. As expected, the recovered biomass (g) from aged media increased with the age of the media, which reached plateau at three days (Figure 5.1). This provides evidence of cell growth in the aged media where cells will metabolise utilising nutrients for growth and secreting waste products. The plateau at three days suggests further incubation results in cell death and lysis, so no significant increase in biomass would be expected after ten days. Similarly, a significant amount of double stranded DNA (ds DNA) was detected in the aged media, suggesting extracellular DNA could be released into the environment through numerous mechanisms including lysis, autolysis and active secretion systems (Ibáñez, Zafra and González-Pastor, 2017). Extracellular DNA has been observed in both the WS and VM biofilm (E. Moshynets, Spiers' Research Group), and this research suggests the highest level of eDNA production is within the first day of growth. Cell death

may also cause increase eDNA in the environment, with higher cell death expected in the three and ten day media, contributing to elevated levels of dsDNA within the system. Other biofilm-promoting products such as cellulose could be present within the aged media, however as aged-media was made with shaking incubation biofilms did not form making the presence of these products less likely.

The pH of the aged media was also compared to provide evidence of chemical changes. As growth period increases, a change in pH is expected to occur, with a higher biomass resulting in an increased change in pH (Ratzke, Jonas and Gore, 2018). This would suggest media aged for longer where more biomass is created would show more significant changes in the environmental pH. In agreement with expectation, the pH of media aged with wild-type SBW25 cells significantly changed, with a longer aging period resulting in a bigger pH change. However, the pH surprisingly increased as the aged of the media increased. Many waste products and secondary metabolites of bacterial metabolisms are organic acids, which would lower the environmental pH as demonstrated in populations of *Paenibacillus* spp. (Ratzke, Jonas and Gore, 2018). The increase in pH found in *P. fluorescens* SBW25 media could be a result of the production of basic pyoverdine proteins, as pyoverdines are heat resistant and so can survive the autoclaving process (Wasserman, 195). Pseudomonads are known to produce iron scavenging siderophore molecules for the essential up-take of free-iron (Cornelis, 2010), and fluorescent pseudomonads produce high affinity siderophores called pyoverdines. *P. fluorescens* SBW25 undergoes pyoverdine-mediated iron uptake, in which 31 genes are suspected to be involved in the biosynthesis, transport and regulation of pyoverdine molecules (Moon *et al*., 2008). Work developing isoelectric focusing to identify and differentiate pyoverdines produced by environmental fluorescent pseudomonads found many basic pyoverdines. *P. fluorescens* ATCC 138525 demonstrated pyoverdines with isoelectric point *(pI)* values between 7.6 and 8.6 (Meyer *et al*., 1998). Mass-spectrometry comparison of pyoverdines produced by *P. fluorescens* SBW25 revealed pyoverdines consisting of a partly cyclic seven residue peptide backbone, identical to *P. fluorescens* ATCC 138525 (Moon *et al*., 2008), suggesting *P. fluorescens* SBW25 also produces basic pyoverdines, possibly contributing to the increase in pH found in aged media. Further research would aim to confirm the basic nature of pyoverdines produced from *P. fluorescens* SBW25 through isoelectric focusing as demonstrated in Meyer *et al*, (1998).

In addition, the composition of KB* media may also contribute to a rise in pH. Recent research found the pH of LB media increased in pH after *E. coli* ATCC 25922, *P. putida* KT2440 and *P. pseudoalcaligenes* CECT 5344 were incubated for 40 h. However, when minimal media was used in the same experiment, where glucose was the sole carbon

source, the pH of the media drops as expected (Sanchez-Clemente, 2018). This suggests the carbon source and by-product from metabolism of carbon substrates can effect the direction of pH change within a system. In addition, the reduction of sulfate present in KB* media could also contribute to the increase in media pH. Under anoxic conditions the reduction of sulfate by bacterial metabolism has been shown to increase pH in culture of microbial communities sampled from acidic lakes (Meier, Piva and Fortin, 2012). This further suggests the composition of KB* media and the associated by-products effects the direction in which environmental pH changes. Future work could explore the change in pH when the media composition is altered, both the carbon substrate and salts present within the *P. fluorescens* SBW25 system.

pH can be considered a public bad, in which all members are harmed in the population (Ratzke, Jonas and Gore, 2018). pH controls microbial processes and reaction rates, and variation within environmental pH can cause a variation in microbial process rate (Jones *et al*., 1994; McCalin *et al.,* 2003). This often results in inhibiting if the pH has increased or decreased out with the optimum range specific for each bacterial species (Russell and Dombrowski, 1980; Jones *et al*., 2009). *P. fluorescens* SBW25 is known to prefer acidic conditions (Huang and Lin, 2020), so It was expected that the higher pH in aged media would have a growth inhibiting effect, resulting in a reduction in the rate of WS mutants arising within the population and effect other microbial process such as biofilm-formation.

As metabolism occurs it would be expected for molecules present within the surrounding media to change. Nutrient up-take would result in depletion and breakdown of nutrient molecules, and waste products and metabolites released would introduce new molecules to the environment. Therefore, FTIR analysis should captures changes and I expected to see changes in the transmittance of light throughout the FTIR spectrum between standard KB* and the aged media. By measuring transmittance of light (%) over a series of wavelengths, changes in transmittance should indicate the addition or reduction of molecules, through more or less molecular bonds present within molecules to absorb light. As expected, significant changes in transmittance was observed, both in a selected section of the spectrum, and when the entire spectrum was compared between all media using principal component analysis (Figure 5.3). In the section of FTIR spectrum randomly selected for further analysis, the three and ten-day aged media show a decrease in the detected transmitted light. This indicates more molecular bonds are present which can absorb light at this series of wavenumbers, suggesting waste products are present.

By quantifying biomass and dsDNA recovered from aged media, and the significant changes found in pH and transmitted light through FTIR analysis, I conclude that the metabolism of wild-type SBW25 has a chemical ecosystem engineering effect. The rapid growth rates of *P. fluorescens* SBW25 in static liquid microcosms not only depletes O_2 within the lower liquid regions, but also chemically alters the environment through the uptake of nutrients and production of waste products. A schematic was created to demonstrate the chemical changes of the environment overtime through ecosystem engineering by wild-type SBW25 cells (Figure 5.15). The resulting changes, in particular the increase in pH, will likely affect the growth and fitness of future lineages, characteristics of the tragedy of the commons (Maclean, 2008). Some intermediate and secondary metabolites can promote growth and initiate cross feeding with neighbouring strains (Estrela, Trisos and Brown, 2012) but are also likely to be toxic (Lau *et al*., 2004). Similarly, the change in pH, often termed 'ecological suicide' (Ratzke, Jonas and Gore, 2018) is likely to inhibit the growth of future lineages of *P. fluorescens* SBW25. This was explored utilising microcosm modified with the addition of aged media.

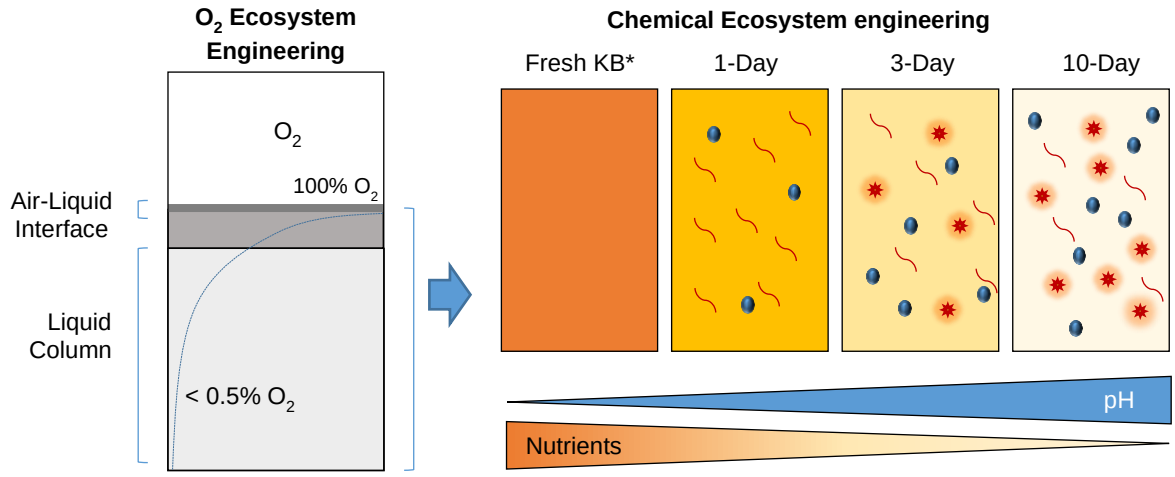


Figure 5.15. Chemical ecosystem engineering in the *Pseudomonas fluorescens* SBW25 system. Initial wild-type SBW25 cells act as ecosystem engineers, depleting O_2 in the lower liquid column of static liquid microcosm, resulting in niche-creating of a high-O_2 region directly below the A-L interface. The metabolism of initial wild-type SBW25 populations also act as chemical ecosystem engineers, through the up-take of nutrients and production of waste products. In the first day, cells release extracellular DNA and metabolic products, and begin to deplete nutrients within the environment. As growth continues nutrients deplete further, and more metabolic and toxic waste products accumulate within the media. This results in an increase in environmental pH, and after three to ten days the media composition is significantly changed.

5.3.2 Environmental modification by wild-type *P. fluorescens* SBW25 effects growth rate and diversification

It is now evidenced that growing populations of wild-type SBW25 do not just act as ecosystem engineers through the depletion of O_2, but also chemically alter KB* media through metabolism. Chemical changes are evident within aging media, with more significant changes found as the media age increases. To asses how these changes impact microbial processes, the chemically altered media was used to investigate the growth and diversification of *P*. fluorescens SBW25.

Initial experiments aimed to investigate the effect of aged media on the evolution of the WS mutant by measuring changes in percentage of WS mutants arising in wild-type SBW25 populations. Chemical changes by ecosystem engineering can alter growth rates of residing strains (McNally and Brown, 2015), so I expected to see a decrease in the number of mutants arising within the population. First, I confirmed the expected percentage of WS mutants over a ten-day period, with WS mutants making up ~ 0.18 % of the population after three days, which increased exponentially to ~ 30% after ten days (Figure 5.4). The percentage of WS mutants dropped in aged media, with a small decrease in the one and three-day aged media, and no WS mutants observed in the ten-day media. This suggests chemical changes in aged media has a growth inhibiting effect on WS mutants arising within the population, caused by the depletion of nutrients and toxic waste products produced by wild-type SBW25. However, within this experiment it was observed that the CFU of wild-type SBW25 populations were also decreasing within aged media (Figure 5.5). The experiment was repeated to determine the effect on wild-type SBW25 growth. As suspected the CFU of wild-type SBW25 significantly decreased as the aged of the media increased. This suggests the ecosystem engineering caused by wild-type SBW25 creates an inhibitory effect on the engineering strain.

P. fluorescens SBW25 favours acidic conditions (Huang and Lin, 2020), with the inhibition of growth in wild-type SBW25 populations likely caused by the increase in pH found in the aged media. Wild-type SBW25 still demonstrates limited growth, suggesting the effect is not as severe as the ‘ecological suicide’ found in populations of *Paenibacillus* spp, however the increase in pH does create a self-inhibiting effect (Ratzke, Jonas and Gore, 2018). In addition to changes in pH, the depletion of nutrient within the system caused by metabolism of initial colonists may also contribute to the decline in wild-type SBW25 growth. KB* media is nutrient rich and when this concentration is decreased, a growth limiting effect is seen (Kassen, Llewellyn and Rainey, 2004, Koza *et al*., 2011, Kuśmierska and Spiers, 2016). *P.*

fluorescens SBW25 has versatile and diverse metabolism, utilising the Enter-Doudoroff pathway in glycolytic metabolism (Huang and Lin, 2020) and the Type III secretion system (TTSS) based on homologue with *P. syringae*. The TTSS secreted proteins are likely to play a role in releasing nutrients for bacterial uptake (Preston, Bertrand and Rainey, 2008) and produce scavenging molecules such as siderophore (Cornelis, 2010). This allows *P. fluorescens* SBW25 to exploit the environment for nutrient up-take, quickly degrading the environment. Initial colonists benefit, however future generations struggle in a nutrient deplete environment. This provides further evidence of tragedy of the commons (Hardin, 1968) occurring within the *P. fluorescens* system, not just through the selfish up-take of O_2, but also through nutrient uptake and degradation of the chemical environment.

The effects of ecosystem engineering often outlast the engineering strain (Hastings *et al*., 2007), affecting the growth of not just future generations, but other residing strains. A decrease in WS mutants arising within the population would suggest a growth inhibiting effect of the chemically altered media. However, as wild-type SBW25 growth is lower in aged media, less WS mutants arising with the population would also be expected. I suspect the decrease of WS mutants in aged media is a combination of the reduction in numbers of wild-type SBW25 caused by self-inhibition, and the chemical changes in the aged media causing an inhibitory effect on WS mutant cells. It is clear chemical changes caused by *P. fluorescens* SBW25 metabolism affects both growth and diversification, however further investigation was required to determine if WS mutants arising within a chemically altered environment are adapted to the surroundings. Three WS isolates were successfully recovered from one-day aged media evolution experiments, allowing for changes and adaption to be explored.

5.3.3 WS mutants evolved in one-day aged media show changes in biofilm characteristics and fitness

Changes in environmental conditions can initiate adaption and evolution in bacterial population, and this has been explored in the *P. fluorescens* SBW25 system. The WS mutant can better exploit the high-O_2 region created by wild-type SBW25 colonists and the Fuzzy-spreader mutant is adapted to growth in the low-O_2 liquid column (Rainey and Travisano, 1998; Ferguson, Bertel and Rainey, 2013). Within WS mutant populations further adaption and changes have been observed when other environmental parameters within the microcosms system have been changed. This includes changes in growth, biofilm-characteristics, colony morphology and fitness when the composition of the media is changed (Kassen, Llewellyn and Rainey, 2004; Venail *et al*., 2011; MacLaughlin, 2016). The

close association with ecological opportunity and adaptive radiation therefore suggests that the WS mutant is sensitive to changes within the environment, and this will affect phenotype and fitness (Koza *et al*., 2017).

From initial evolution experiments, three WS isolates were successfully recovered from wild-type SB25 populations incubated for three days in one-day aged media (aged media – WS mutants, AM-WS mutants). I expected the AM-WS mutants would demonstrate significant differences in colony morphology, biofilm characteristics and fitness as a result of arising in an environment which has been previously chemically altered through wild-type SBW25 metabolism. It was also expected that these characteristics would change when compared in standard KB* media and one-day aged media, with an expectation that WS mutants recovered from aged media would perform better in aged media, as a result of adaption to the environment in which diversification took place.

All three AM-WS mutants demonstrated notably smaller colony morphology on KB* plates (Figure 5.6). Only three isolates were recovered from the one-day media, so a small sample size may create bias in the results of the recovered AM-WS mutants. However, small colony morphology was consistent across all three mutants. Colony morphology can be an indicator of phenotypic variation which is known to be an important adaptive process to overcome changes and stressors within the environment. Changes in colony size, texture and colour has been demonstrated as a result of media composition in populations of different *Pseudomonas aeruginosa* strains (Sousa *et al.*, 2013). Similar colony patterns have been observed in different systems, in particular during the diversification of bacterial population in static liquids, where large wrinkled colony morphology is found in A-L interface biofilm-forming mutants in many other pseudomonad populations including *P. putida* KT2240 (Bridier, Piard and Bouchez, 2019), *P. aeruginosa* PA01 and PA14 (Boles, Thoendel and Singh, 2004; Flynn *et al.,* 2016) and in other genres including the gram-positive *B. subtilis* 3610 (Dragos *et al*., 2018). Future work would aim to determine if reduction in colony size of the WS mutant was consistent over all aged media, and if the change in colony size is linked to a specific environmental change or adaption. This could be the increase in pH or one of the many metabolites produces by wild-type SBW25. From this, further exploration could determine if this is a strain specific change or if a similar effect is seen in the many A-L interface biofilm-forming wrinkled mutants found in different species of pseudomonads and *B. subtilis* 3610, suggesting similar colony patterns in response to environmental change. Changes in the colony morphology of WS mutants evolved in one-day aged media suggest that changes may also be observed in biofilm-characteristics and fitness. The combined

biofilm assay can be used to differentiate between WS mutant phenotypes (Koza *et al*., 2017). In standard KB* microcosms the biofilm characteristics of the AM-WS were lower than WS mutants recovered from KB* media (Figure 7.1). This suggest that an additional one-day aging period of the environment is sufficient to create significant phenotypic differences to the WS mutant. However, despite a decrease in biofilm characteristics in AM-WS in fresh media, I still expected AM-WS mutants would perform better in the one-day media.

Adaption to a new and changing environment creates new phenotypes and genetics within a bacterial population. This increases the diversity and divergence within a population, and as a result increases bacterial survival (Yachi and Loreau, 1999; Boles *et al*., 2004).This suggests the phenotypic changes found in the AM-WS may indicate adaption to a new environment, therefore would outperform the archetypal WS in the one-day aged media. The combined biofilm assay was repeated with the AM-WS mutants and the archetypal WS in both fresh KB* microcosms and one-day aged microcosms (Figure 5.8). Growth in the one-day aged media was significantly lower in all WS mutants, suggesting all mutants are subject to a growth inhibiting effect of the aged media. However, attachment was higher in all WS mutants in one-day media, which may reflect the increase ds DNA in the aged media. DNA present in the aged media is likely extracellular DNA (eDNA, Confocal microscopy findings by O. Moshynets, Spiers' Research Group) and strains of *P. aeruginosa* have been demonstrated to utilise eDNA for biofilm attachment (Nivens *et al.,* 2001).However, the AM-WS mutants show lower attachment and strength than the archetypal WS mutant in both media types. This indicates that the AM-WS mutants do not have improved biofilm characteristics in the aged media compared to the archetypal WS mutants and is still subject to the inhibition of environmental modification. However, as previously demonstrated in Chapter three with the WS mutants, VM biofilm and CBFS mutant, increased biofilm characteristics did not lead to improved fitness within static liquid microcosms. The less robust biofilm characteristics of the AM-WS could still be a sign of adaption, perhaps conservation of energy by limiting biofilm production as a result of a depleted environment. To further investigate, the fitness of the AM-WS mutants were investigated.

Competitive fitness (W), calculated as the ratio of Malthusian Parameters (Lenski *et al*., 1991) was used to compare the fitness of the archetypal WS and the AM-WS mutants against the non-biofilm-forming *P. fluorescens* SG70 (Figure 5.10). The AM-WS mutants evolved under static conditions in media aged for one-day with wild-type SBW25 prior to inoculation. It would therefore be expected that the AM-WS mutants would have increased fitness in this environment compared to the archetypal WS mutant, demonstrating adaption.

The AM-WS mutants had increased fitness in the one-day aged media compared to the archetypal WS, suggesting the AM-WS are better adapted to growth and survival within this media. However, the competitive fitness of the AM-WS mutants was significantly higher in fresh KB* media. This could reflect the inhibiting effect AM-WS mutants face in the one-day media, or adaption to a growth limiting environment provides further competitive fitness in the standard KB* environment. Research suggests mutations can occur across a number of different loci within a common pathway in the WS mutant, with mutations evident in the multiple genes within the *wsp* operon leading to the wrinkly spreader phenotype (Bantanki *et al*., 2007; Mcdonald *et al*., 2009; Lind, Farr and Rainey 2015; Udall *et al.,* 2015). This results in the disruption of cyclic-*di*-GMP homeostasis, which can lead to fitness changes by variation in substrate utilisation patterns (MacLean and Bell 2003; MacLean, Bell and Rainey 2004). I suggest environmental stressors such as altered nutrient availability and waste product accumulation may result in the phenotypic variation found in the AM-WS, which could be caused by mutations within a different loci within the *wsp* operon leading to a change in fitness an possible substrate utilisation. Further genetic analysis would aim to compare where mutations have occurred within AM-WS mutants, to determine if changes in the chemical environmental through ecosystem engineering causes variation in the loci of the wrinkly spreader mutation.

The initial aim of this section, prior to the COVID-19 campus closure, was to obtain WS mutants from all aged media, which would allow further investigation of the effect of ecosystem engineering. This would allow a comparison of growth, biofilm characteristics and fitness in media aged for longer. I would expect to see a greater change in the WS mutant phenotype when evolved in the three and ten-day aged media, as chemical analysis suggests further metabolism has occurred resulting in less nutrients available and greater built up of waste products. Initial evidence from the evolution experiments within aged media also suggested that WS mutants arising within a chemically altered environment were genetically unstable, which has been previously demonstrated in WS mutants on agar plates (Spiers, 2007). This presented difficulties in successfully selecting WS mutants in the three-day aged media which reverted back to wild-type colonies after selection and re-streaking on to fresh KB* plates. In addition, wild-type SBW25 growth was significantly lower in three and ten-day media, therefore the number of WS mutants arising within the population would also be expected to fall. Future experiments would require significantly higher replication to obtain WS mutants from the three and ten-day media, and if still no mutants appeared in the ten-day media this would suggest that the chemically changes within this media completely inhibits the diversification of wild-type SBW25. However, the effect of the three and ten-day

aged media could still be explored utilising the archetypal WS, to give an insight into the effect of a prolonged media aging period on the characteristics and fitness of the WS mutant.

5.3.4 The biofilm characteristics and fitness of the archetypal WS mutant changed significantly as the age of the modified media increased

I have previously demonstrated that an initial aging period of one-day causes significant changes to the growth, biofilm characteristics and fitness of both AM-WS mutants and the archetypal WS. Further analysis could not investigate AM-WS mutants from older media, however I can still explore the effect of aging media on the archetypal WS mutant growth, characteristics and fitness. This will demonstrate the effects of an increased aging period. As further chemical changes are seen in the three and ten-day aged media, I would expect to see a further drop in the growth, biofilm characteristics and fitness of the WS mutants caused by an increase in metabolic waste products from wild-type SBW25, and less nutrient availability.

The biofilm characteristics of the WS mutants were significantly altered in all aged media, with a greater decrease in growth, attachment and strength found in the three and ten-day media (Figure 5.11). Little biofilm development was observed in the ten-day media, reflected with a mean strength of 0.04 g, suggesting almost complete inhibition of biofilm development. In agreement with previous experiments, attachment in one- day media was significantly higher than other media types, reflected in the increase extracellular DNA present in the envrionment. A recent investigation identified extracellular DNA produced by *P. fluorescens* SBW25 (O. Moshynets, A. Kayumov, S. Rymar and A. Spiers, unpublished observations), in agreements within observations of *P. aeruginosa* PA01 biofilms (Whitechurch *et al*., 2002). In *P. aeruginosa* PA01 eDNA increases cell surface and adhesive properties, increasing attachment in biofilm development (Nivens *et al*., 2001; Whitechurch *et al*., 2002; Bouffartigues *et al*., 2015). This suggests that the WS mutant can benefit from the production of eDNA by wild-type SBW25 after a one-day period. However, a further media aging period results in a drop in ds DNA, and further nutrient depletion and toxic metabolites production dominates creating an inhibiting effect to biofilm attachment. As hypothesised, an increased aging period of the media resulted in greater changes in the biofilm characteristics of the WS mutant.

One main effect of ecosystem engineering is the effect of fitness on neighbouring species. Often the engineering species can better respond to the changing environment and outcompete neighbouring species (Callahan, Fukami and Fisher, 2014). However, initial

growth experiments demonstrate wild-type SBW25, the engineering strain, also suffers inhibition from the depleted environment, suggesting a tragedy of the commons or ecological suicide effect (Maclean, 2008; Ratzke, Jonas and Gore, 2018). Within static conditions the WS mutant has a known fitness advantage over wild-type SBW25, by increased access to O_2 through biofilm-formation. This advantage is lost in a shaken environment, where biofilm-formation cannot occur, and WS mutants waste energy on cellulose production (Spiers *et al*., 2002; Green *et al*., 2011; McDonald *et al*., 2011; Lind, Farr and Rainey, 2015). Pair-wise fitness comparison between the wild-type SBW25 and WS mutant can indicate which strain is better adapted to chemical environmental change, in both static and shaken conditions where the WS mutants obtains and loses competitive fitness advantage respectively.

Despite the inhibition of biofilm-formation in the WS mutant, the WS mutant competitive fitness increased in static incubation conditions. The converse was found in shaken conditions, where the WS mutant does not have a competitive fitness in fresh KB* media, and fitness significantly decreased as the age of the media increased (Figure 5.13). The competitive fitness of the WS mutant was negatively corelated between static and shaken conditions (Figure 5.14), as the age of the media increases, the static fitness increases and the shaken fitness decreases. This suggests that in a static environment, the WS mutant can still form a biofilm even if characteristics are altered, so can still receive increased O_2 access compared to wild-type SBW25. Wild-type SBW25 not only has lower O_2 access but is in the liquid column where the toxic metabolites are present. Biofilm-formation at the A-L interface may result in a proportion of the WS mutant population avoiding contact with the toxic metabolites, achieving higher population growth. In shaken, both are subjected to toxic waste products, and the WS mutant can no longer form a biofilm increasing access to O_2, and energy expenditure for EPS and cellulose production is wasted (Spiers *et al*., 2002; Green *et al*., 2011; McDonald *et al*., 2011; Lind, Farr and Rainey, 2015). Here nutrient availability becomes the dominant limiting resources, and toxic waste products further contribute to growth inhibition. This is in agreement with previous research into the effect of toxic metabolites on fitness in laboratory populations (Travisano 1997). Even in controlled laboratory conditions bacteria alter the environment through metabolic waste, and although may not be completely lethal to bacteria to the extent of ecological suicide (Ratzke, Jonas and Gore, 2018), it may affect the viability of cells within this environment, effecting both the bacteria that produce them and neighbouring cells.

This research has confirmed that wild-type SBW25 cells not only act as ecosystem engineers through the depletion of O_2 in the lower liquid column, but also act as chemical ecosystem engineers significantly altering the chemical composition of the media through

the depletion of nutrient and production of metabolic waste products. This results in inhibition of growth in wild-type SBW25, but also effects the biofilm characteristics and fitness of WS mutants within the population, and this effect is increased as the age of the media increases. Further work would look to determine the exact cause of inhibition, through metabolite identification. It is already known that nutrient concentration effects population growth *in P. fluorescens* SBW25, but future work would aim to identify specific metabolites and waste products that contribute to the inhibition of growth and biofilm-formation in WS mutants. Many secondary metabolites of *P. fluorescens* SBW25 have been identified (Leisinger and Margraff, 1979), however a main issue with metabolite identification is the ability to separate and distinguish intra-cellular metabolites and extra-cellular metabolites (Villas-Boas and Bruheim, 2008). Further chemical analysis or metabolomics (Mallick *et al*., 2019; Pinu and Villas-Boas, 2016) of the aged media could help understand key metabolites released that alter the chemical environment resulting in the inhibition of cells within the environment. Metabolite identification can also lead to possible agricultural or pharmaceutical applications (Zhao *et al.,* 2020; Shahid, Malik and Mehnaz, 2018), with key interest into molecules that cause growth and biofilm inhibition.

5.4 Conclusion

P. fluorescens SBW25 acts as an ecosystem engineer, through the depletion of O_2 within the liquid column of static liquid microcosms, resulting in the creation of a high-O_2 ecological niche at the top of the liquid column, directly below the A-L interface (Koza *et al*.,2017). However, bacterial cells can alter the surrounding environment in many ways, most common through metabolism, resulting in nutrient depletion and production of metabolic waste products. This research demonstrates chemical ecosystem engineering by initial wild-type SBW25 colonists, where metabolic activity significantly alters the chemical composition of the media, most notably increasing environmental pH. Media aged for one to ten days inhibited growth of wild-type SBW25 and WS mutants cells, further evidence of a tragedy of the commons effect in static liquid microcosms. Media aged by wild-type SBW25 cells significantly altered diversification, and the percentage and phenotype of WS mutants arising within the population, as demonstrated in one-day aged media. As the aging period increased, further significant changes were found in the WS mutant biofilm characteristics and fitness. However, WS mutants evolved in a depleted environment were better adapted than the archetypal WS, suggesting adaption to nutrient depletion and waste-product accumulation.

This chapter has provided further insight into the ecological dynamics occurring within static liquid microcosms. Chemical ecosystem engineering occurs through bacteria metabolism, which has significant effect on the growth, evolution, biofilm characteristics and fitness of both the engineering strain and other residing strains. This demonstrated the importance of studying the surrounding environment in microbial studies, where metabolic activity is inevitable, likely altering the environment. This research also exhibits how quickly key microbial processes such as growth and biofilm-formation can change as a result of cells modifying their environment and can provide a fresh perspective in understanding changes in microbial infections and ecological important microbial processes.

Chapter 6. Thesis Discussion and Conclusion

6.1. Steps and applications of successful air-liquid interface biofilm-formation

Many species of bacteria can from biofilms at the air-liquid interface of static liquid columns in experimental microcosms, although it still remains unclear where A-L interface biofilms might occur in nature (Kovács and Dragoš, 2019). Within the *Pseudomonas fluorescens* SBW25 system A-L interface biofilm-formation has be extensively researched (Spiers, 2014; Koza *et al*., 2017), however, fundamental questions surrounding the need for A-L interface biofilm-formation, and why it is more successful than a non-biofilm-forming aerotaxis based strategy remain. The research presented in this thesis confirms A-L interface biofilm-formation is more successful at localising and maintaining cells within high-O_2 region than aerotaxis motility, and WS mutants are better able to access the A-L interface by lower surface tension through the production of surface-active-agents and penetrate the interface. This allows biofilm-formation to occur above the interface, resulting in dry surface wrinkled biofilms. In static liquid microcosms cells are faced with physical displacement, through bioconvection currents and Brownian motion, which constantly displace planktonic cells down the liquid column. A-L interface biofilm-formation prevents cells from requiring continued aerotaxis motility and can position cells at the high-O_2 region without further movement.

Other species of bacteria also demonstrate similar diversification, where A-L interface biofilm mutants arise in static populations of *Bacillus subtilis* 3610 (Dragos *et al*., 2018), *P. aeruginosa PA01 and PA14* (Boles, Thoendel and Singh, 2004; Flynn *et al.,* 2016), and *P. putida* KT2440 (Bridier, Piard and Bouchez, 2019). Similar to the tensiometer analysis carried out in this research, pendant drop tensiometry has compared the elasticity and viscosity of A-L interface biofilms of *P. fluorescens* SBW25, *B. subtilis* PY79 and NCIB 3610 and *E. coli* K12 *Csr* in response to changes in environmental conditions (Rühs et al., 2013). However, little research investigates changes in interface interactions within diversifying populations. Future work could apply surface tension and cell localisation analysis to other species or strains with A-L interface biofilm-forming adaptive mutants to determine if the ability to lower surface tension and increase cell localisation to the high-O_2 region in static liquid microcosm is a common trait with biofilm-forming mutants. If common amongst successful A-L interface biofilm mutants, this could be a useful tool to enhance A-L interface biofilm-formation for industrial or medical use.

A-L interface biofilm-formation can have unique biotechnology or biomedical applications (Saichana *et al.,* 2015). In industrial settings A-L interface biofilms have application to bioremediation and vinegar production*. Methylobacterium hispancium* EM2 A-L interface biofilms are shown to remove Pb(II) in contaminated drinking water (Jeong *et al*., 2019) and the ability to form 'floating' A-L interface biofilm-formation increase thermotolerance for acetic acid strains involved in vinegar production (Saichana *et al*., 2015). In addition, many polysaccharides associated with A-L interface biofilm matrix, in particular cellulose, are shown to be applicable to biomedical settings. The cellulose matrix structure of *Gluconacetobacter xylinus* DSM 2325 is similar to collagen when grown in membrane bioreactors (Hofinger, Bertholdt and Weuster-Botz, 2011), and other microbial cellulose use has been applied as a novel skin biological dressing for burns or tissue regeneration (Fontana *et al*., 1990; Sajjad *et al*., 2019). Findings from my research could enhance A-L interface biofilm-formation in other species, increasing matrix yield, by assessing how species access the A-L interface, and increase the ability to lower surface tension to achieve more successful biofilm-formation. I also demonstrated by limiting the effect of physical displacement, both non-biofilm-forming and biofilm-forming strains can increase localisation to the high O_2 region. This could be applied to increase the biomass of A-L interface biofilms for industrial or biomedical use, by increasing media viscosity.

6.2 Strong biofilm-formation is not necessarily an indication of fitness success

Within the *P. fluorescens* SBW25 system it is clear biofilm-forming WS mutants form strong biofilms and have a fitness advantage over non-biofilm forming strains, achieving increased cell localisation to the high-O_2 region. Throughout my research I have been interested in what happens below the biofilm, and how strains utilise the entire environment including the low-O_2 liquid column. This revealed WS mutant loses its fitness advantage when competing with other biofilm-forming mutants from the SBW25 lineage. Weaker biofilm formers with lower strength and attachment levels, such as the VM biofilm produced by wild-type cells, has a fitness advantage over the WS mutant, and results suggest this is because wild-type cells can maximise productivity by colonising both the A-L interface through biofilm-formation and the lower liquid column with planktonic cells. Similar results were found in the soil-wash multi-species microcosms. Although biofilm characteristics did increase over time in community serial-transfer experiments, attachment and strength levels were still moderate compared to biofilms formed by the WS mutants. The serial-transfer experiment selected for plastic phenotypes, which could be considered generalists, forming A-L interface biofilms and colonising the low-O_2 liquid column. I suggest this provides a fitness advantage in complex community microcosms. Similarly, WS mutants evolved in aged media as a results

of wild-type SBW25 ecosystem engineering form weaker biofilms than the archetypal WS mutant, however demonstrate increased competitive fitness.

Evaluating literature surrounding success and strong biofilm-formation, some research areas conclude strong biofilm-forming strains dominant. In particular in food control settings, where strong biofilm-forming strains of *Staphylococcus aureus* prevail and show increased resistance to antimicrobial agents (Ou *et al*., 2020). This is perhaps as a result of intensive disinfection processes, where strong biofilm-formers can persist and survive. However, other research agree with my findings, suggesting strong biofilms are not necessarily an advantage. Strong biofilm-forming pathogenic *Escherichia coli* strains demonstrate little advantage over weaker biofilm-formers in relation to antibiotic resistance, virulence and other associated bacterial traits such as the ability to produce siderophores (Naves *et al*., 2008) and strong biofilm-formation has little association with the presence of drug resistance and genes associated with virulence in clinical isolates of *Enterococcus* spp. (Shridhaar and Dhanashree, 2019). Similarly hospital-acquired *Acinetobacter baumannii* infections found strong biofilm producing strains were generally more susceptible to antibiotics, in which authors suggest protection provided by biofilm characteristics result in the loss of mechanisms responsible for resistance of planktonic cells (Krysciak et al., 2017).

Throughout this research I have shown an association with weaker to moderate biofilm-formation and the ability to colonise the under-utilised low-O_2 niche, providing a fitness advantage at both the population and community level. This may indicate the persistence and antibiotic resistance of moderate and weak biofilm-forming pathogenic strains in infections as a result of strains maximising productivity and survival by colonising both biofilm-associated and non-biofilm space. Similarly, as Krysciak *et al*. (2017) suggests, these strains can retain mechanisms responsible for resistance in planktonic cells, and benefit from biofilm-lifestyle where matrix substance and mobilisation of resistant plasmids through horizontal gene transfer increases resistance of biofilm-forming cells.

Throughout this research the development of a cell localisation assay has shown differences in cell density within the lower liquid column between strains and communities, where colonisation helps maximise productivity. Many microcosm-based assays, such as the competitive fitness assays, includes cells and growth throughout the whole system. The cell localisation assay was able to differentiate the high-O_2 region and liquid column when comparing changes in productivity. Future work could look to create a similar approach at comparing difference in fitness between two strain competing within the biofilm region, or the

liquid column region. This may provide insight to how changes in biofilm characteristics affects fitness within the biofilm region.

6.3 The influence of non-biofilm space and plastic phenotypes

Air-liquid interface biofilm-formation has been well characterised in many model bacterium (Koza 2014; Kovács and Dragoš, 2019; Flynn *et al.,* 2016) but little is known about the influence of liquid column space beneath the biofilm. In static liquid microcosms the liquid column makes up more than 90% of the total ecosystem, therefore previous studies showing changes in growth or fitness have only taken into consideration <10% of the environment where the biofilm is situated. Throughout this research I have shown the importance of colonisation within the liquid column, where plastic phenotypes capable of A-L interface biofilm-formation and liquid column colonisation can help maximise productivity in community microcosms. This fitness advantage was also found in competitive fitness assays, where other strains within the SBW25 lineage capable of colonising both regions but produced weaker biofilms out-competed the WS mutant which has little liquid column colonisation. Finally, the suggestion of migration between the A-L interface biofilm and liquid column in serial-transferred static liquid microcosms highlights the importance of studying non-biofilm space within a biomedical and industrial setting, where strains in non-biofilm space are capable of re-establishing biofilms after disturbance.

Antibiofilm therapeutics focus on the disruption and dispersal of biofilm communities through the use of natural products, nanoparticles and disrupting biofilm-formation related processes such as cell-cell communication (quorum sensing) (Harrison *et al*., 2020; Lu *et al*., 2019; Chaudhary et al., 2020).However, less attention is paid to the non-biofilm space and how this can impact microbial community success. Development of biofilm therapeutics is common, where species diversity and taxonomic characterisation method development is focussed on biofilm associated space in which subsequent treatment is made (Mandakhalikar *et al*., 2018; Suzuki, Yoshida and Nakano, 2005). Investigating the wider ecosystem in biofilm-related infections could improve biofilm treatment, by limiting the re-establishment by neighbouring cells unaffected by initial biofilm treatment or disruption. Similarly, industrial processes such as bioremediation and waste-water treatment focus on enhancing biofilm properties, including genetic engineering, improving chemotactic ability and use of synergistic mixed species biofilms (Singh, Paul and Jain, 2006). Bioremediation and biotransformation of organic and pharmaceutical pollutants and heavy metal contamination focus on the benefits of biofilm structure and durability (Edwards and Kjellerup, 2013). Here, plastic phenotypes could be incorporated, particular in contaminated

water settings, where community productivity could be maximised by colonising non-biofilm space beneath the A-L interface.

Plastic phenotypes are expected to be found in highly fluctuating environments (Van ben Bergh *et al.,* 2018) and are likely to present many difficulties in biofilm treatment. It acts as a bet-hedging approach (Beaumont *et al.,* 2009), thought to be among the earliest evolutionary solutions to life in fluctuating environments. Cells in non-biofilm space may survive biofilm targeted therapeutics, but similarly cells situated within the biofilm are better protected from antimicrobial agents through protection from EPS and horizontal gene transfer obtaining resistant genes. The ability to form in both the biofilm and planktonic state also ensures mechanism sometimes lost in adaption to biofilm life-style can be retained within the planktonic populations (Krysciak et al., 2017). While plastic phenotypes, like generalists, may have lower fitness in a particular environment compared to specialised phenotypes arising with the population (Van ben Bergh *et al.,* 2018), the highly fluctuating conditions of many infection and industrial settings demonstrate their importance in insuring microbial community survival.

6.4 Ecosystem engineering and inevitable changes in microbial communities

Metabolic activity is a naturally occurring process in microbial communities, and within this research I explored the significant effects metabolic waste products and nutrient depletion has on the development and key processes with bacteria populations and communities, known as ecosystem engineering. Within the *Pseudomonas fluorescens* SBW25 system growth of initial wild-type colonists significantly alter the environment, with chemical changes in addition to O_2 depletion detected after only one-day of growth. These chemical changes not only inhibited growth of future wild-type SBW25 cells but affect the diversification and characteristics of WS mutants arising within the population. The effects of ecosystem engineering were also apparent within multi-species community microcosms, where nutrient depletion and toxic waste accumulation limit community productivity creating further selection pressures within the system.

Recent research seeks to understand the ecological role of bacteria metabolites (Kiesewalter *et al*., 2020), with many focussing on identifying the specific role of secondary metabolites, concluding they can be utilised as signalling molecules (Romero *et al.*, 2011) or weapons for competition (Foster and Bell, 2012). New computational and experimental approaches are being developed to enhance our understanding of the effects of metabolic activity and metabolic interactions within microbiome studies, aiming to develop predictable

models to manipulate microbiome interactions taking into account metabolic interactions (Antoniewicz, 2020). Investigating the effects of ecosystem engineering through metabolic activity within the *Pseudomonas fluorescens* SBW25 and multi-species communities concludes regardless of the role of bacteria metabolites, the natural process of metabolism creates change within the environment. This occurs within a short time period and can create further selection pressure within the environment, affecting ecological and evolutionary dynamics with populations and communities. Where there is opportunity and growth within microbial communities, over time there will be change in characteristics and function.

The role and application of microbial communities is endless, with application in soil remediation for plant growth enhancement (Rumble and Gange, 2017), microbial fuel cells (Borello *et al*., 2020), heavy metal bioremediation (Jin *et al*., 2018) and food production, in particular the ripening of cheese (Ndoye *et al*., 2011). Here research focuses on the enhancement of microbial community activity over time, with an aim of achieving a stable community taking into account environmental, chemical and biological factors to increase efficiency. The significant effects of metabolic ecosystem engineering throughout this research reiterates the significant changes in evolutionary process and community function caused by the environmental modification of microbes, suggesting no microbial community is truly stable. The diversification or change in one community member can upset any homeostasis within the community, including adaption to a competitive phenotype or changes in metabolic activity resulting in different metabolites and waste entering the system and affecting neighbouring species. This can be used to our advantage, where understanding change can be used to better adapt or change the community to increase and maximise efficiency, however this must be constantly reviewed as community or populations change. Ecological and evolutionary succession studies continue to be vital in our understanding of how and why microbial populations and communities change through natural occurring processes.

6.5 Concluding remarks

Air-liquid interface biofilm-formation is a successful colonising strategy, capable of localising cells to the high-O_2 region in static liquid microcosm presenting a fitness advantage over non-biofilm forming strategies, in particular aerotaxis. However, the ability to colonise both the A-L interface through biofilm-formation and the low-O_2 liquid column is advantageous, where biofilm characteristics may be weaker, but utilising the entire ecosystem maximises productivity. This was demonstrated at both the population and community level, where

plastic phenotypes dominated community microcosms in fluctuating short-term evolution experiments. The retention of phenotypic plasticity in community microcosms highlight the importance in studying the entire ecosystem, in which non-biofilm space can influence the productivity and survivability of microbial communities in biomedical and industrial settings. Further exploration of the ecological dynamics within the microcosm model system demonstrated the ability of microbial communities and populations to change over a short time period, where the natural processes of metabolic activity constantly changes the environment, creating opportunity and selective pressures within the system. Where there is opportunity microbes continually adapt to survive, changing resulting community properties and ecological dynamics within.

Experimental eco-evolutionary studies continue to reveal answer the underlying questions and mechanisms surrounding biofilm-formation and development, and by developing multi-species systems to reflect the complexity of natural occurring driving microbial adaption and survival. Here we can apply new knowledge to understanding the progression of pathogen associated infections, changes in ecologically important communities and how to apply microbial communities to reverse the effects of anthropogenic pressures.

Appendices

Appendix 1: Supplementary Information Chapter 4

A1.1 Productivity and biofilm-associated traits changes throughout serial-transfer

The combined biofilm assay was used to measure biofilm characteristics between each transfer throughout the serial-transfer experiment. Analysis compared the first and final microcosms in each transfer as productivity (OD_{600}), attachment (A_{570}) and strength (grams) fluctuated throughout the ten transfers (Figure A1.1 – A1.3).

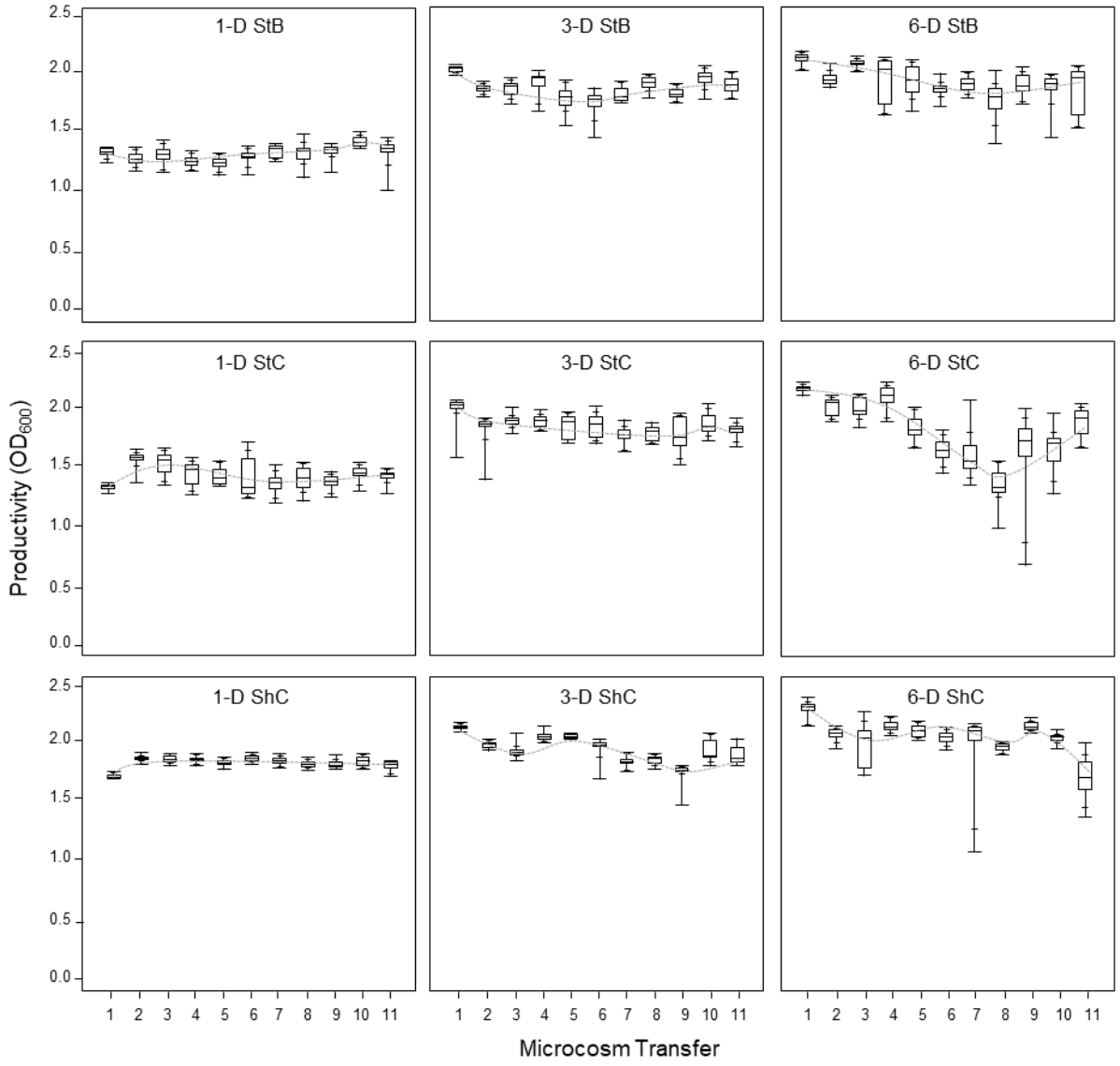


Figure A1.1 Productivity thorughout serial-transfer of soil-wash community. Community productivity (OD_{600}) fluctuates in the one, three and six day serial-transfer experiments. Boxplots are shown. Trend lines are descriptive only.

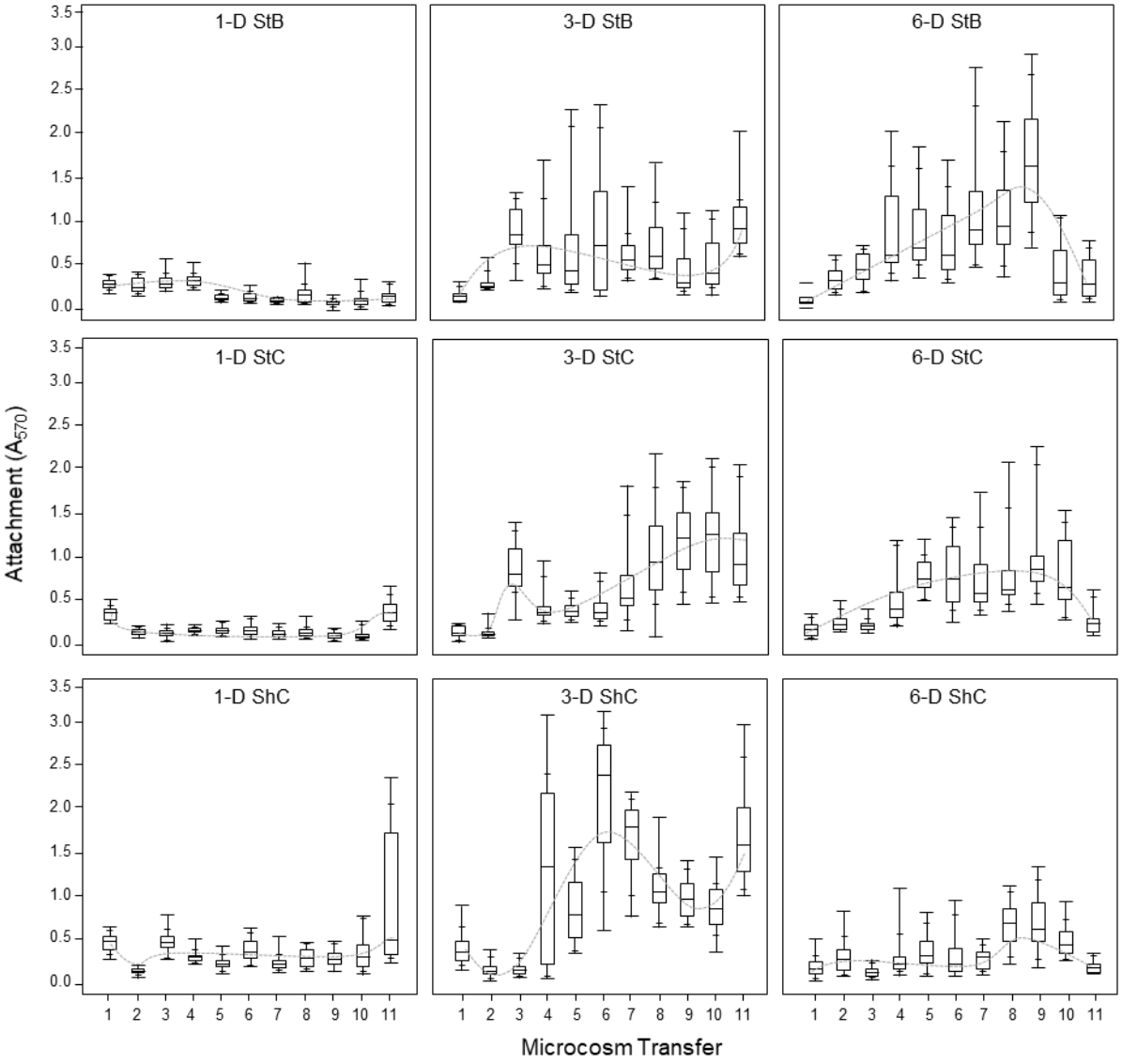


Figure A1.2 Biofilm attachment thorughout serial-transfer of soil-wash community. Biofilm attachment (A_{570}) fluctuates in the one, three and six day serial-transfer experiments. Boxplots are shown. Trend lines are descriptive only.

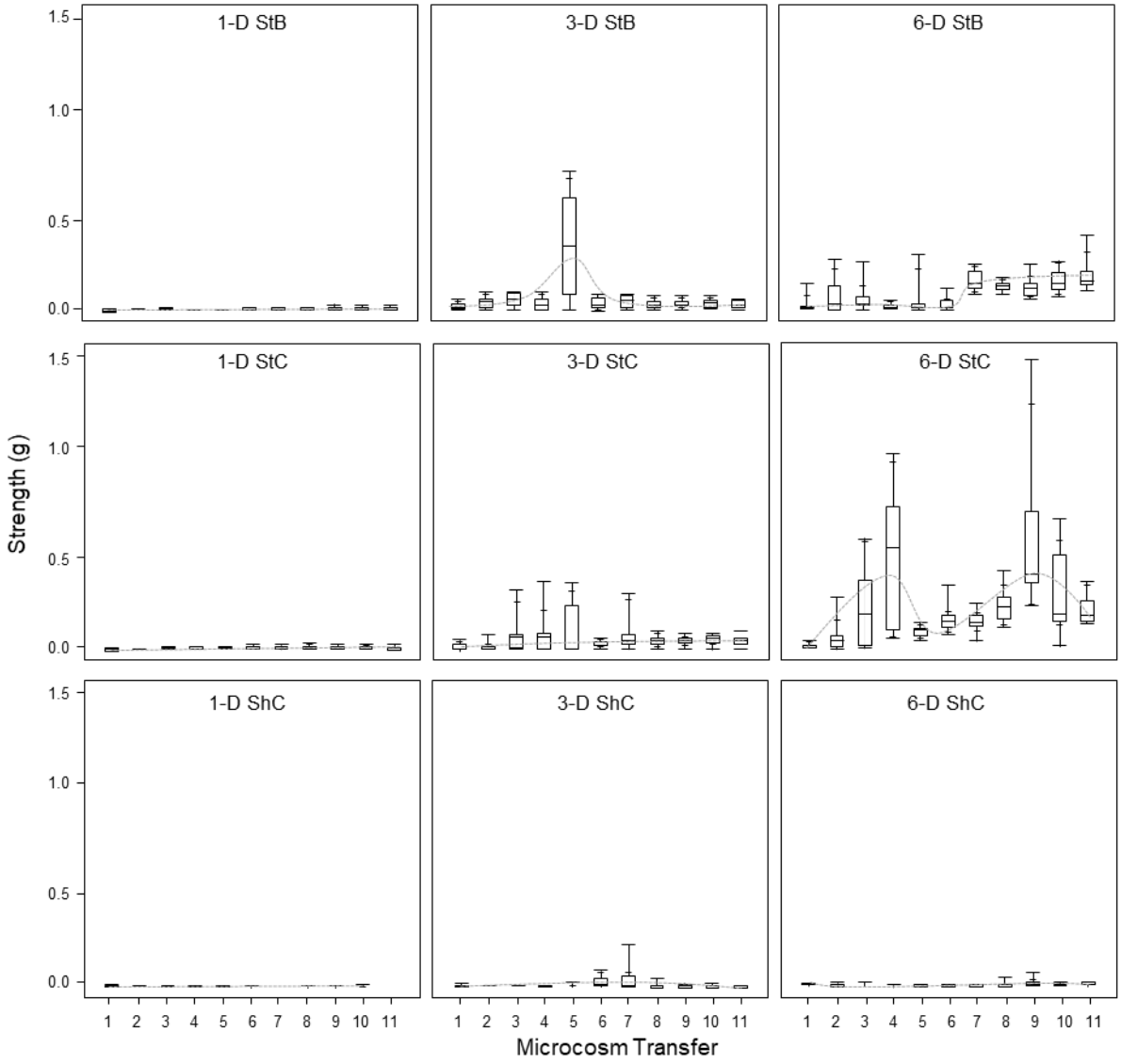


Figure A1.3 Biofilm strength thorughout serial-transfer of soil-wash community. Community strength (grams) fluctuates in the one, three and six day serial-transfer experiments. Boxplots are shown. Trend lines are descriptive only.

A1.2 Confirmatory modelling analysis for community productivity changes

To confirm mixed effects (random) model of productivity found in chapter 4 (Table A1.1 and A1.2), confirmation models were performed with outlier analysis. Outlier analysis was performed, and residuals were remove until data fitted a normal distribution (Anderson-Darling P = 0.104 (a more updated version of JMP was utilised for modelling analysis which replaces the Shapiro-Wilk's test with the Anderson-Darling, a more robust test)). This model confirmed that incubation period (one, three & six days), incubation conditions (shaken & static) and shift (from the first to the last incubation) had a significant effect on community

productivity, but also suggests that sample type (community or biofilm) is also significant (See table 7.1 for model summary). Significant differences were found within effects and interactions, revealed using LS Means Differences Student's t and Tukey HSD (a = 0.05). Interactions included incubation period (1 day < 3 day < 6 day; Q = 2.3), conditions (static < shaken), shift (final < initial), sample type (biofilm < community), period x conditions (1 day/static < 1 day/shaken < 3 day/static < 3 day/shaken < 6 day/static < 6 day/shaken; Q = 2.8), period x sample type (1 day/biofilm < 1 day/mixed-community < 3 day/mixed-community & 3 day/biofilm < 6 day/biofilm & 6 day/mixed-community; Q = 2.8) and incubation period x shift (1 day/initial < 1 day/final < 3 day/final < 6 day/final < 3 day/initial < 6 day/initial).

Table A1.1. Modell summary for initial and final productivity transfer data with outlier analysis.

GLMM RSquared = 0.99

Fixed Effects	P - Value	F	**Interactions**	P - Value	F	**Random Effects**	% Variance
Incubation period (days)	0.000	$F_{2.2}$ = 4816	Incubation period * initial or final microcosm	0.000	$F_{2.2}$ = 778	Community replicate	7.28
Initial and final microcosm	0.000	$F_{1.1}$ = 822	Incubation period * incubation conditions	0.000	$F_{2.2}$ = 557	Microcosms replicate [community replicate]	1.8
Incubation conditions	0.000	$F_{1.1}$ = 2008	Incubation period * sample type	0.24	$F_{2.2}$ = 1.4		
Sample type	0.016	$F_{1.1}$ = 6					

To confirm mixed effects (random) model of productivity utilising the biofilm strength and attachment data found in chapter 4 (Table 4.2), confirmation models were also performed with outlier analysis. Outlier analysis was performed, and residuals were removed until data fitted a normal distribution (Anderson-Darling P = 0.08). This model confirmed that biofilm

attachment (A_{570}) was significant, but also suggests that biofilms strength (grams) is significant (See table 4.2 for model summary). Significant differences were found within effects and interactions, revealed using LS Means Differences Student's t and Tukey HSD (a = 0.05). Interactions included incubation period (1 day < 3 day < 6 day; Q = 2.3), sample type (biofilm < community), incubation period x conditions (1 day/static < 1 day/shaken < 3 day/ static < 3 day/shaken & 6 day/static < 6 day/shaken; Q = 2.8), incubation period and sample type (1 day/biofilm < 1 day/mixed-community < 3 day/mixed-community & biofilm < 6 day/ mixed-community & biofilm; Q = 2.8).

Table A1.2. Modell summary for initial and final productivity data and biofilm attachment and strength with outlier analysis.

GLMM RSquared = 0.99

Fixed Effects	P - Value	F	**Interactions**	P - Value	F	**Random Effects**	% Variance
Incubation period (days)	0.000	$F_{2.2}$ = 1057	Incubation period * incubation conditions	0.000	$F_{2.2}$ = 322	Community replicate	5.6
Initial and final microcosm	0.000	$F_{1.1}$ = 22	Incubation period * sample type	0.04	$F_{2.2}$ = 3.08	Microcosms replicate [community replicate]	0.0
Incubation conditions	0.000	$F_{1.1}$ = 1714	Incubation period * initial and final microcosm	0.000	$F_{2.2}$ = 96		
Sample type	0.98	$F_{1.1}$ = 5.2	Incubation period * Attachment	0.95	$F_{2.2}$ = 0.04		
Attachment	0.0007	$F_{1.1}$ = 11.5	Incubation period * Strength	0.02	$F_{2.2}$ = 3.8		
Strength	0.005	$F_{1.1}$ = 7.7	Attachment* Strength	0.0001	$F_{1.1}$ = 15.6		

A1.3 Individual isolate-level cell distribution

Cell distribution was characterised for individual isolates from the initial soil-wash and six-day transfer communities. Eight isolates were characterised for each community which show variation in cell distribution, with differences in cell localisation to the high-O_2 region in static liquid microcosms.

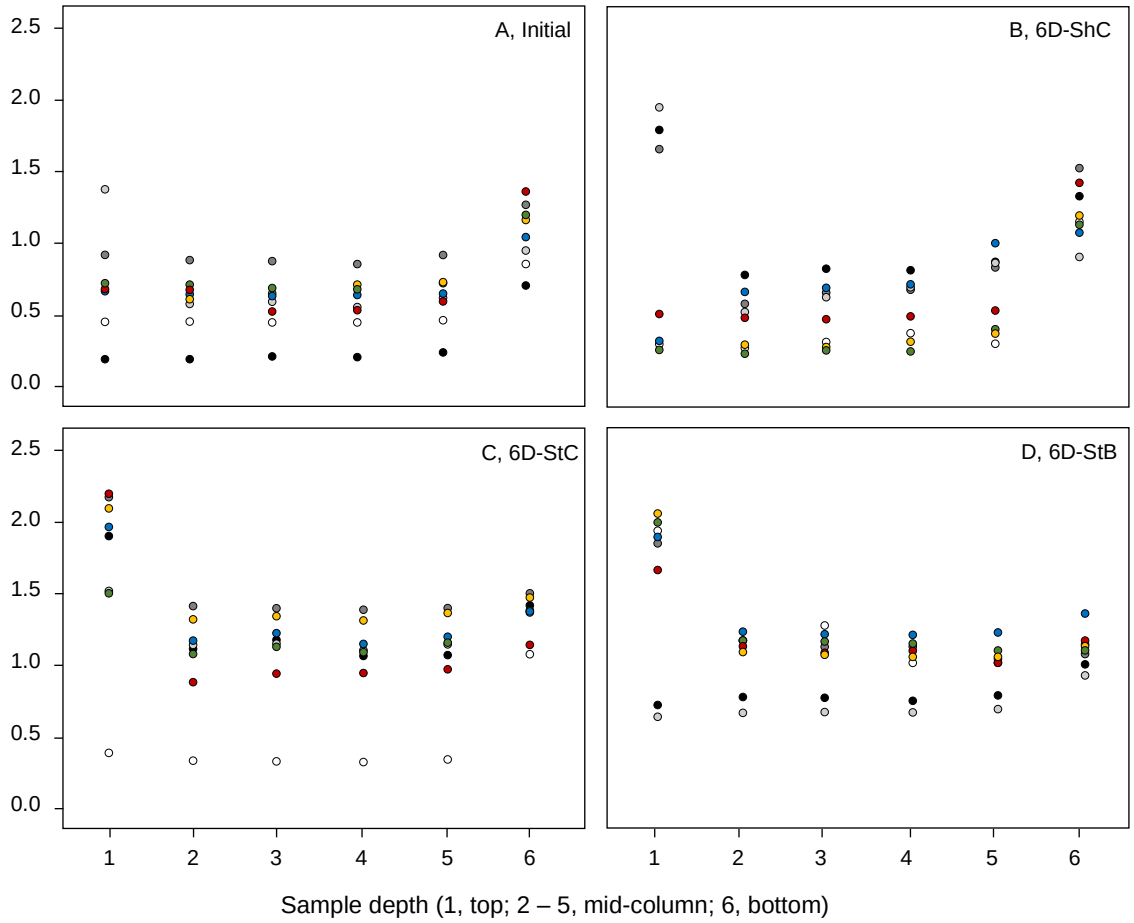


Figure A1.4. Individual isolate cell distribution in initial soil-wash and six-day transfer communities. Cell distribution (OD_{600}) throughout the liquid column was explored by measuring cell density for every 1 ml sequentially down through the liquid column, where the first ml contains the biofilm community. Community isolates (n = 8) show variation in cell distribution and localisation to the high-O_2 region (Panel A – initial soil-wash, Panel B – six-day shaken community transfer, Panel C – six-day static community transfer, Panel D – six-day static biofilm transfer). Means ± SE (n = 5) are shown, but for clarity error bars smaller than the symbol size are not shown.

A1.4 Isolate Swimming motility

Plate based motility assays were undertaken with isolates (n = 8) from the initial soil-wash and six-day transfer communities. Replicate inoculations were made (n = 4) and the swimming diameter (cm) was measured after 48 h (Table A1.3). The maximum diameter based on plate size and two inoculations per plate was 5.2 cm, and a diameter below 1 cm was indicative of random cell diffusion and not swimming motility.

Table A1.3. Swimming diameter (cm) of initial soil-wash and six-day transfer community isolates.

Community	SW	6-D ShC	6-D StC	6-D StB
Swimming Diameter (cm)				
Isolate 1	5.2 ± 0.0	0.7 ± 0.0	5.2 ± 0.0	3.1 ± 0.1
Isolate 2	3.9 ± 0.3	0.7 ± 0.0	3.2 ± 0.1	3.3 ± 0.1
Isolate 3	3.5 ± 0.1	3.7 ± 0.2	3.1 ± 0.1	4.2 ± 0.3
Isolate 4	3.4 ± 0.4	3.5 ± 0.1	3.3 ± 0.2	3.3 ± 0.1
Isolate 5	3.6 ± 0.2	3.8 ± 0.2	2.9 ± 0.1	4.1 ± 0.3
Isolate 6	5.2 ± 0.0	0.7 ± 0.0	3.2 ± 0.2	3.2 ± 0.1
Isolate 7	5.2 ± 0.0	3.2 ± 0.2	3.3 ± 0.3	4.1 ± 0.4
Isolate 8	0.7 ± 0.0	3.4 ± 0.1	3.2 ± 0.1	3.3 ± 0.2

Appendix 2: Publications

Jerdan, R, Cameron, S, Donaldson, E, Lungin, O, Moshynets, O and Speirs, A. (2020) 'Community biofilm-formation, stratification and productivity in serially-transferred microcosms', *FEMS Microbiology Letters*, (Accepted/In press).

Publication from research chapter 4. Main experimenter, experimental design and data analysis, manuscript preparation and supervision of visiting group members.

ACCEPTED MANUSCRIPT

Community biofilm-formation, stratification and productivity in serially-transferred microcosms

Robyn Jerdan, Scott Cameron, Emily Donaldson, Olga Iungin, Olena V Moshynets, Andrew J Spiers ✉

FEMS Microbiology Letters, fnaa187, https://doi.org/10.1093/femsle/fnaa187
Published: 18 November 2020

Abstract

The establishment of O_2 gradients in liquid columns by bacterial metabolic activity produces a spatially-structured environment. This produces a high-O_2 region at the top that represents an un-occupied niche which could be colonised by biofilm-competent strains. We have used this to develop an experimental model system using soil-wash inocula and a serial-transfer approach to investigate changes in community-based biofilm-formation and productivity. This involved ten transfers of mixed-community or biofilm-only samples over a total of 10–60 days incubation. In all final-transfer communities the ability to form biofilms was retained, though in longer incubations the build-up of toxic metabolites limited productivity. Measurements of microcosm productivity, biofilm-strength and attachment levels were used to assess community-aggregated traits which showed changes at both the community and individual-strain levels. Final-transfer communities were stratified with strains demonstrating a plastic phenotype when migrating between the high and low-O_2 regions. The majority of community productivity came from the O_2-depleted region rather than the top of the liquid column. This model system illustrates the complexity we expect to see in natural biofilm-forming communities. The connection between biofilms and the liquid column seen here has important implications for how these structures form and respond to selective pressure.

Koza, A, Jerdan, R, Cameron, S and Spiers, A. (2020) 'Three biofilm types produced by a model pseudomonad are differentiated by structural characteristics and fitness advantage', Microbiology, 166(8).

Publication utilising cell localisation assay developed in this research. Equal experimenter, experimental design and data analysis and manuscript development.

MICROBIOLOGY Volume 166, Issue 8

Research Article

Three biofilm types produced by a model pseudomonad are differentiated by structural characteristics and fitness advantage

Anna Koza[1], Robyn Jerdan[1], Scott Cameron[1], Andrew J. Spiers[1]

View Affiliations

First Published: 10 June 2020 | **https://doi.org/10.1099/mic.0.000938**

Model bacterial biofilm systems suggest that bacteria produce one type of biofilm, which is then modified by environmental and physiological factors, although the diversification of developing populations might result in the appearance of adaptive mutants producing altered structures with improved fitness advantage. Here we compare the air–liquid (A–L) interface viscous mass (VM) biofilm produced by *Pseudomonas fluorescens* SBW25 and the wrinkly spreader (WS) and complementary biofilm-forming strain (CBFS) biofilm types produced by adaptive SBW25 mutants in order to better understand the link between these physical structures and the fitness advantage they provide in experimental microcosms. WS, CBFS and VM biofilms can be differentiated by strength, attachment levels and rheology, as well as by strain characteristics associated with biofilm formation. Competitive fitness assays demonstrate that they provide similar advantages under static growth conditions but respond differently to increasing levels of physical disturbance. Pairwise competitions between biofilms suggest that these strains must be competing for at least two growth-limiting resources at the A–L interface, most probably O_2 and nutrients, although VM and CBFS cells located lower down in the liquid column might provide an additional fitness advantage through the colonization of a less competitive zone below the biofilm. Our comparison of different SBW25 biofilm types illustrates more generally how varied biofilm characteristics and fitness advantage could become among adaptive mutants arising from an ancestral biofilm–forming strain and raises the question of how significant these changes might be in a range of medical, biotechnological and industrial contexts where diversification and change may be problematic.

Received: 25/02/2020 Accepted: 05/05/2020 Published Online: 10/06/2020

Keyword(s): adaptive radiation , air–liquid (A–L) interface biofilms , competitive fitness , experimental evolution , microcosms , P. fluorescens SBW25 , Pseudomonas and wrinkly spreader

Jerdan, R, Lungin, O, Moshyents, O, Potters, G and Spiers, A. (2020) 'Extending an eco-evolutionary understanding of biofilm-formation at the air-liquid interface to community biofilms', In *Bacterial biofilms.* InTech Publishers.
Book chapter including work from chapter 3 and publication Jerdan *et al*. (2019) and unpublished data from chapter 4. Involved in manuscript development.

Chapter

Extending an Eco-Evolutionary Understanding of Biofilm-Formation at the Air-Liquid Interface to Community Biofilms

Robyn Jerdan, Olga Iungin, Olena V. Moshynets, Geert Potters and Andrew J. Spiers

Abstract

Growing bacterial populations diversify to produce a number of competing lineages. In the *Pseudomonas fluorescens* SBW25 model system, Wrinkly Spreader mutant lineages, capable of colonising the air-liquid interface of static microcosms by biofilm-formation, rapidly appear in diversifying populations with a fitness advantage over the ancestral wild-type strain. Similarly, a biofilm is rapidly produced by a community containing many biofilm-competent members, and selection by serial transfer of biofilm samples across microcosms results in a gradually changing community structure. Both the adaptive radiation producing Wrinkly Spreaders and the succession of biofilm communities in these static microcosms can be understood through evolutionary ecology in which ecological interactions and evolutionary processes are combined. Such eco-evolutionary dynamics are especially important for bacteria, as rapid growth, high population densities and strong selection in the context of infections can lead to fast changes in disease progression and resistance phenotypes, while similar changes in community function may also affect many microbially mediated biotechnological and industrial processes. Evolutionary ecology provides an understanding of why bacterial biofilms are so prevalent and why they are such a successful colonisation strategy, and it can be directly linked to molecular analyses to understand the importance of pathways and responses involved in biofilm-formation.

Keywords: adaptive radiation, air-liquid (A-L) interface biofilms, evolutionary ecology, experimental evolution, fitness, microcosms, oxygen gradients, *Pseudomonas*, Wrinkly Spreaders

Jerdan, R, Kusmierska, A, Petric, M and Speirs, A. (2019) 'Penetrating the air-liquid interface is the key to colonization and wrinkly spreader fitness', *Microbiology,* 165(10).

Publication from research chapter 3. Main experimenter, experimental design and data analysis, manuscript preparation and supervision of visiting group members.

MICROBIOLOGY

RESEARCH ARTICLE

Jerdan *et al.*, *Microbiology* 2019;165:1061–1074

DOI 10.1099/mic.0.000844

MICROBIOLOGY SOCIETY

Penetrating the air–liquid interface is the key to colonization and wrinkly spreader fitness

Robyn Jerdan[1], Anna Kuśmierska[1,2], Marija Petric[1] and Andrew J. Spiers[1,*]

Abstract

In radiating populations of *Pseudomonas fluorescens* SBW25, adaptive wrinkly spreader (WS) mutants are able to gain access to the air–liquid (A–L) interface of static liquid microcosms and achieve a significant competitive fitness advantage over other non-biofilm-forming competitors. Aerotaxis and flagella-based swimming allows SBW25 cells to move into the high-O_2 region located at the top of the liquid column and maintain their position by countering the effects of random cell diffusion, convection and disturbance (i.e. physical displacement). However, wild-type cells showed significantly lower levels of enrichment in this region compared to the archetypal WS, indicating that WS cells employ an additional mechanism to transfer to the A–L interface where displacement is no longer an issue and a biofilm can develop at the top of the liquid column. Preliminary experiments suggest that this might be achieved through the expression of an as yet unidentified surface active agent that is weakly associated with WS cells and alters liquid surface tension, as determined by quantitative tensiometry. The effect of physical displacement on the colonization of the high-O_2 region and A–L interface was reduced through the addition of agar or polyethylene glycol to increase liquid viscosity, and under these conditions the competitive fitness of the WS was significantly reduced. These observations suggest that the ability to transfer to the A–L interface from the high-O_2 region and remain there without further expenditure of energy (through, for example, the deployment of flagella) is a key evolutionary innovation of the WS, as it allows subsequent biofilm development and significant population increase, thereby affording these adaptive mutants a competitive fitness advantage over non-biofilm-forming competitors located within the liquid column.

References

Abrams, P. (2001) ‘Modelling the Adaptive Dynamics of Traits Involved in Inter- and Intraspecific Interactions: An Assessment of Three Methods’, Ecology Letters, 4, pp.166-167. doi:10.1046/j.1461-0248.2001.00199.x.

Adler, J. (1973) ‘A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by Escherichia coli’, *Journal of General Microbiology*, 74(1), pp.77-91. doi:10.1099/00221287-74-1-77.

Alder, M. *et al*. (2012) ‘Studies of bacterial aerotaxis in a microfluidic device’ *Lab on Chip*, 12 (22), pp.4835-4847. doi:10.1039/c2lc21006a.

Allison, S. and Martiny, J. (2008) ‘Resistance, resilience and redundancy in microbial communities’, *PNAS*, 105, pp.11512-11519. doi:org/10.1073/pnas.0801925105.

Almås, A., Mulder, J. and Bakken, L. (2005) 'Trace Metal Exposure of Soil Bacteria Depends on Their Position in the Soil Matrix', *Environmental Science and Technology*, 39(16), pp.5927-5932. doi:org/10.1021/es048113w.

Alsohim, S. *et al.* (2014) ‘The biosurfactant viscosin produced by *Pseudomonas fluorescens* SBW25 aids spreading motility and plant growth promotion’, Environmental Microbiology, 16 (7), pp.2267-2281. doi:10.1111/1462-2920.12469.

Altermatt, F. *et al.* (2015) ‘Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution’, *Methods in Ecology and Evolution*, 6(2), pp.218-231. doi:org/10.1111/2041-210X.12312.

Amann, R. and Fuchs, B. (2008) ‘Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques’, *Nature Reviews Microbiology*, 6, pp.339-348. doi:org/10.1038/nrmicro1888.

Amann, R., Ludwig, W. and Schleifer, K. (1995) ‘Phylogenetic identification and in situ detection of individual microbial cells without cultivation’, *Microbiology Reviews*, 59(1), pp.143-169.

Andre, M., Dufour, D. and Rainey, P. (2019) 'Causes and Biophysical Consequences of Cellulose Production by Pseudomonas fluorescens SBW25 at the Air-Liquid Interface', *Journal of Bacteriology*, 201(18), doi:10.1128/JB.00110-19.

Angell, P. (1999) ‘Understanding microbially influenced corrosion as biofilm-mediated changes in surface chemistry’, *Current Opinion in Biotechnology*, 10(3), pp.266-272.

Anotniewicz, M. (2020) ‘A guide to deciphering microbial interactions and metabolic fluxes in microbiome communities’, *Current Opinion in Biotechnology*, 64, pp.230-237. doi:org/10.1016/j.copbio.2020.07.001.

Arcerenza, L. (2016) ‘Constraints, Trade-offs and the Currency of Fitness’, *Journal of Molecular Evolution,* 82 (2-3), pp.117-127. doi:10.1007/s00239-016-9730-3.

Ardizzone, S. *et al.* (2001) ‘Microcrystalline cellulose suspensions: effects on the surface tension at the air–water boundary’, *Colloids and Surfaces*, 176, pp.239-244. doi:10.1016/S0927-7757(00)00672-5.

Armisen, R and Galatas, F. (2009) ‘*Agar’,* in Phillips, G and Williams, P (2nd ed) *Handbook of colloids*. Cambridge: Wood-head publishing Ltd, pp.83-203.

Armitano, J., Méjean, V. and Jourlin-Castelli, C. (2014) ‘Gram-negative bacteria can also form pellicles’, *Environmental Microbiology*, 6(6), pp.534-544. doi:org/10.1111/1758-2229.12171.

Asahi, Y. *et al*. (2015) ‘Simple observation of *Streptococcus* mutants biofilm by scanning electron microscopy using ionic liquids’, *AMB Express*, 5(6). doi:org/10.1186/s13568-015-0097-4.

Awasthi, A. *et al*. (2014) ‘Biodiversity acts as insurance of productivity of bacterial communities under abiotic perturbations’, *The ISME Journal*, 9(5). doi:10.1038/ismej.2014.91.

Bachmann, H. *et al.* (2017) ‘Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria’, *FEMS Microbiology Reviews*, 41, pp.201-219. doi:10.1093/femsre/fux024

Bailey, M. and Thompson, I. (1992) ‘Detection systems for phyllosphere Pseudomonads’, in Wellington, E and van Elsas, J. (ed.) *Genetic Interactions Between Microorganisms in the Natural Environment*. Oxford: Pergamon Press, pp.127-141

Bantinaki, B*. et al*. (2007) ‘Adaptive divergence in experimental populations of Pseudomonas fluorescens. III. Mutational origins of Wrinkly Spreader diversity’, *Genetics*, 176(1), pp.441-453. doi:org/10.1534/genetics.106.069906.

Barak, R. *et al*. (1982) ‘Aerotactic response of Azospririllum brasilense’, *Journal of bacteriology*, 152(2), pp.643-649.

Beaumont, H. *et al*. (2009) ‘Experimental evolution of bet hedging’, *Nature*, 462, pp.90-93. doi:org/10.1038/nature08504.

Beier, S. *et al*. (2015) ‘Phenotypic plasticity in heterotrophic marine microbial communities in continuous culture’, *The ISME Journal*, 9, pp.1141-1151. doi:org/10.1038/ismej.2014.206.

Bell, G. (2007) ‘Selection: The mechanism of Evolution’ Oxford UK: Oxford university press.

Berg, H. (1993) ‘Movement of self-propelled objects’, in Berg, H (ed.). *Random walks in biology*, Expanded edition. Princetown: Princetown University Press, pp.75-93.

Berg, H. (2003) ‘*E. coli in Motion*’, New York, NY: Springer-Verlag.

Berg, J. (2009) ‘Fluid interfaces and capillarity’ in Berg, J. *An Introduction to Interfaces and Colloids*, Singapore: World Scientific Publishing Co, pp. 23-106.

Berger, W. and Parker, F. (1970) ‘Diversity of Planktonic Foraminifera in Deep-Sea Sediments’, *Science*, 168(3937), pp.1345-1347. doi:10.1126/science.168.3937.1345.

Besemer, K. *et al*. (2007) ‘Biophysical Controls on Community Succession in Stream Biofilms’, *Applied and Environmental Microbiology*, 73, pp.4966-4974. doi:10.1128/AEM.00588-07.

Besemer, K. *et al*. (2012) ‘Unravelling assembly of stream biofilm communities’, *The ISME Journal*, 6, pp.1459-1468. doi:org/10.1038/ismej.2011.205.

Bjarnsholt, T. *et al*. (2009) ‘*Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients’, *Paediatric Pulmonology*, 44(6), pp.547-558. doi:org/10.1002/ppul.21011.

Blattner, G. *et al*. (1997) 'The complete genome sequence of Escherichia coli K-12', *Science,* 277(5331) pp.1453-1462. doi:10.1126/science.277.5331.1453.

Blount, Z., Borland, C. and Lenski, R. (2008) 'Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*', *PNAS,* 105(23), pp.7899-7906. doi:org/10.1073/pnas.0803151105.

Boer, W. (1991) 'Nitrification at Low pH by Aggregated chemolithotrophic Bacteria', *Applied and Environmental Microbiology*, 57(12), pp.3600-3604.

Boles, B., Thoendel, M. and Singh P. (2004) 'Self-generated diversity produces ''insurance effects'' in biofilm communities', *PNAS,* 101(47), pp.16630-16635. doi:10.1073/pnas.0407460101.

Boogert, N., Paterson, D. and Laland, K. (2006) 'The implication of Niche construction and ecosystem engineering for conservation biology', *BioScience*, 56(7) pp.570-578. doi:org/10.1641/0006-3568(2006)56[570:TIONCA]2.0.CO;2.

Borello, D. *et al*. (2020) 'Use of microbial fuel cells for soil remediation: A preliminary study on DDE', *International Journal of Hydrogen Energy*, in press.

Bouffartigues, E. *et al*. (2015) 'The absence of the Pseudomonas aeruginosa OprF protein leads to increased biofilm formation through variation in c-di-GMP level', *Frontiers in Microbiology,* 6(630)*,* doi:10.3389/fmicb.2015.00630.

Bowler, O. (1989*) Evolution, the history of an idea*. (2nd edn) London: University of California press, LtD.

Boyle, K. *et al*. (2013) 'Exploiting social evolution in biofilms', *Current opinion in Microbiology*, 16(2), pp.207-212. doi:org/10.1016/j.mib.2013.01.003.

Branda, S. (2001) 'Fruiting body formation by *Bacillus subtilis*', *PNAS*, 98(20), pp.11621-11626. doi:org/10.1073/pnas.191384198.

Breidenstein, E., de la Fuente-Núñez, C. and Hancock, R. (2011) '*Pseudomonas aeruginosa*: all roads lead to resistance', *Trends in Microbiology*, 19, pp.419-426. doi:10.1016/j.tim.2011.04.005.

Bren, A. and Eisenbach, M. (2000) 'How signals are heard during bacterial chemotaxis: protein-protein interactions in sensory signal propagation', *Journal of Bacteriology*, 182(42), pp.6865-68673. doi:10.1128/JB.182.24.6865-6873.2000.

Bridier, A., Piard, J. and Bouchez, T. (2019) 'Emergence of a synergistic diversity as a Response to Competition in *Pseudomonas putida* biofilms', *Microbial Ecology*, 80, pp.47-59. doi:10.1007/s00248-019-01470-z.

Brocker, C., Thompson, D. and Vasiliou, V. (2012) 'The role of hyperosmotic stress in inflammation and disease', *Biomolecular Concepts*, 3(4), pp.345-364. doi:org/10.1515/bmc-2012-0001.

Brockhurst, M. *et al.* (2014) 'Running with the Red Queen: the role of biotic conflicts in evolution', *Proceedings of the Royal Society B,* 281(1797). doi:org/10.1098/rspb.2014.1382.

Brockhurst, M. *et al*. 'Niche Occupation Limits Adaptive Radiation in Experimental Microcosms', *PLoS ONE*, 2(2), e193. doi:org/10.1371/journal.pone.0000193.

Brockhurst, M. *et al.* (2006) 'Character Displacement Promotes Cooperation in Bacterial Biofilms', *Current Biology*, 16(20), pp.2030-2034. doi:org/10.1016/j.cub.2006.08.068.

Brockhurst, M. *et al*. (2010) ''Ecological drivers of the evolution of public-goods cooperation in bacteria', *Ecology,* 91(2), pp.334-340. doi:org/10.1890/09-0293.1.

Brockhurst, M., Buckling, A. and Gardner, A. (2007) 'Cooperation Peaks at Intermediate Disturbance', *Current Biology*, 12(9), pp.761-765. doi:org/10.1016/j.cub.2007.02.057.

Brockhurst, M., Colegrave, N. and Hodgson, D. (2007) 'Buckling A. Niche occupation limits adaptive radiation in experimental microcosms', *PLoS ONE*, 2(2), e193, doi:org/10.1371/journal.pone.0000193.

Buckling, A and Rainey, P. (2002) 'Antagonistic coevolution between a bacterium and a bacteriophage', *Proceedings of the royal society B*, 269(1494), doi:org/10.1098/rspb.2001.1945.

Buckling, A. *et al*. (2003) 'Adaption Limits Diversification of Experimental Bacterial Populations', *Science*, 302(5653), pp.2107-2109. doi:10.1126/science.1088848.

Buckling, A. *et al*. (2009) 'The *Beagle* in a bottle', *Nature*, 457, pp.824-829. doi:org/10.1038/nature07892.

Buell, C. *et al.* (2003). 'The complete sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000', *PNAS*, 100(18), pp.10181-10186. doi:org/10.1073/pnas.1731982100.

Burmølle, M. *et al*. (2006) 'Enhanced Biofilm Formation and Increase Resistance to Antimicrobial Agents and Bacterial Invasion are Caused by Synergistic Interactions in Multispecies Biofilms', *Applied Environmental microbiology*, 72, pp.3916-3923. doi:10.1128/AEM.03022-05.

Burmølle, M. *et al*. (2007) 'Establishment and early succession of a multispecies biofilm composed of soil bacteria', *Microbial Ecology*, 54, pp.352-362. doi:10.1007/s00248-007-9222-5.

Callahan, B., Fukami, T. and Fisher, D. (2014) 'Rapid evolution of adaptive niche construction in experimental microbial populations', *Evolution*, 68(11), pp.3307-3316. doi:10.1111/evo.12512.

Castledine, M., Buckling, A. and Padfield, D. (2019) 'A shared coevolutionary history does not alter the outcome of coalescence in experimental populations of *Pseudomonas fluorescens'*, *Journal of Evolutionary Biology*, 32(1), pp.58-65. doi:org/10.1111/jeb.13394.

Chang, W. *et al*. (2007) 'Alginate Production by *Pseudomonas putida* creates a Hydrated Microenvironment and Contributes to Biofilm Architecture and Stress Tolerance under Water-Limiting Conditions', *Journal of Bacteriology*, 189(22), pp.8290-8299. doi:10.1128/JB.00727-07.

Chaudhary, S. *et al.* (2020) 'Role of Nanoparticles as Antibiofilm Agents: A Comprehensive Review', Current Trends in Biotechnology and Pharmacy, 14(1), pp.97-110. doi:10.5530/ctbp.2020.1.10.

Chen, M., Yu, Q. and Sun, H. (2013) 'Novel Strategies for the Prevention and Treatment of biofilm related infections', *International journal of molecular sciences*, 14(9), pp.18488-18501. doi:org/10.3390/ijms140918488.

Chevin L-M. (2011) 'On measuring selection in experimental evolution', *Biology Letters*, 7(2), pp.210-213. doi:org/10.1098/rsbl.2010.0580.

Chevin, L-M., Lande, R. and Mace, G. (2010) ‘Adaptation, plasticity, and Extinction in a Changing Environment: Towards a Predictive Theory’, *PLoS Biology* 8(4), e1000357. doi:org/10.1371/journal.pbio.1000357.

Christensen, B. *et al*. (2002) ‘Metabolic commensalism and competition in a two-species microbial consortium’, *Applied Environmental Microbiology*, 68, pp.2495-2502. doi:10.1128/AEM.68.5.2495-2502.2002.

Ciofu, O. *et al*. (2000) ‘Chromosomal β-lactamase is packaged into membrane vesicles and secreted from *Pseudomonas aeruginosa’*, *Journal of Antimicrobial Chemotherapy*, 45(1), pp.9-13. doi:org/10.1093/jac/45.1.9.

Clements, F. (1916) *Plant Succession: An Analysis of the Development of Vegetation*. Washington, D.C.: Carnegie Institution of Washington.

Cooper, D. and Goldenberg, B. (1987) ‘Surface-Active Agents from Two *Bacillus* species’, *Applied and Environmental Microbiology*, 53(2), pp.224-229.

Cooper, V., Bennett, A. and Lenski, R. (2001) ‘Evolution of thermal dependence of growth rate of *Escherichia coli* populations during 20,000 generations in a constant environment’, *Evolution*, 55(5), pp.889-896. doi:org/10.1111/j.0014-3820.2001.tb00606.x.

Cornelis, P. (2010) ‘Iron uptake and metabolism in pseudomonads’, *Applied Microbiology and Biotechnology*, 86, pp.1637-1645. doi:10.1007/s00253-010-2550-2.

Costerton, J. *et al*. (1994) ‘Biofilms, the customized microniche’, *Journal of Bacteriology*, 176(8), pp.2137-2142. doi:10.1128/jb.176.8.2137-2142.1994.

Costerton, J. *et al*. (1995) ‘Microbial Biofilms’, *Annual Review of Microbiology*, 49, pp.711-745. doi:org/10.1146/annurev.mi.49.100195.003431.

Coughlan, L. *et al*. (2016) ‘New weapons to Fight old enemies: Novel Strategies for the (Bio)control of Bacterial Biofilm in the Food Industry’, *Frontiers in Microbiology,* 7, 1641. doi:org/10.3389/fmicb.2016.01641.

Couvert, O. *et al*. (2019) ‘Modelling the effect of oxygen concentration on bacterial growth rates’, *Food Microbiology*, 77, pp.21-25. doi:org/10.1016/j.fm.2018.08.005.

D’Souza, G. *et al.* (2018) ‘Ecology and evolution of metabolic cross-feeding interactions in bacteria’, *Natural Product Reports*, 35, pp.455-488. doi:10.1039/C8NP00009C.

Dallinger, W. (1878) ‘On the life-history of a minute septic organism: with an account of experiments made to determine its thermal death point’, *Proceeding of the Royal Society of London*, 27, pp.332-350. doi:org/10.1098/rspl.1878.0055.

Darwin, C. (1859) ‘On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races’, in Murray, J. *In the Struggle for life*. London, UK.

Davey, M. and O'Tooles, G. (2000) ‘Microbial biofilms: from Ecology to Molecular Genetics’, *Microbiology and Molecular biology Reviews*, 64(4), pp.847-867. doi:10.1128/MMBR.64.4.847-867.2000.

Davies, D. and Geesey, G. (1995) ‘Regulation of the Alginate Biosynthesis Gene *alg*C in *Pseudomonas aeruginosa* during Biofilm Development in Continuous Culture’, *Applied and Environmental Microbiology*, 61(3), pp.860-867.

Day, R., Laland, K. and Odling-Smee, J. (2003) ‘Rethinking adaptation. The niche-construction perspective’, *Perspectives in Biology and Medicine*, 46(1), pp.80-95, doi:10.1353/pbm.2003.0003.

de Bruijn, I. *et al*. (2007) ‘Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species’, *Molecular Microbiology*, 63(2), pp.417-428. doi:10.1111/j.1365-2958.2006.05525.

De Roy, K. *et al.* (2014) ‘Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities’, *Environmental Microbiology*, 16(6), pp.1472-1481. doi:10.1111/1462-2920.12343.

De Weert, S. *et al.* (2002) ‘Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonisation by *Pseudomonas fluorescens*’, *Molecular plant microbe interactions,* 15 (11), pp.1173-1180. doi:10.1094/MPMI.2002.15.11.1173.

Dean, A., Dykhuizen, D. and Hartl, D. (1986) ‘Fitness as a function of β-galactosidase activity in *Escherichia coli*’, *Genetical Research*, 48(1), pp.1-8. doi:org/10.1017/S0016672300024587.

Deeraksa, A. *et al*. (2005) ‘Characterization and spontaneous mutation of a novel gene, *polE*, involved in pellicle formation in *Acetobacter tropicalis* SKU1100’, *Microbiology,* 151(12), pp.4111-4120. doi:org/10.1099/mic.0.28350-0.

Dirk van Elsas, J. *et al*. (2012) ‘Microbial diversity determine the invasion of soil by a bacterial pathogen’, *PNAS*, 109(4), pp.1159-1164. doi:org/10.1073/pnas.1109326109.

Dobell, C. (1960) *Antony van Leeuwenhoek and his “Little animals”* New York: Dover Publications INC.

Dobzhanksy, T. (1951) *Genetics and the Origin of Species.* New York: Columbia university Press.

Doekes, H., de Boer, R. and Hermsen, R. (2019) ‘Toxin production spontaneously becomes regulated by local cell density in evolving bacterial populations’, *PLOS Computational Biology*, 15(8), e1007333. doi:org/10.1371/journal.pcbi.1007333.

Douterelo, I., Fish, K. and Boxall, J. (2018) 'Succession of bacterial and fungal communities within biofilms of a chlorinated drinking water distribution', *Water Research*, 141, pp.74-85. doi:org/10.1016/j.watres.2018.04.058.

Dragos, A. *et al*. (2018) ‘Evolution of exploitative interactions during diversification in *Bacillus subtilis* biofilms’, *FEMS Microbiology Ecology*, 94(1), fix155. doi:10.1093/femsec/fix155.

Dubey, G. and Ben-Yehuda, S. (2011) ‘Intercellular nanotubes mediate bacterial communication’, *Cell*, 144(4), pp.590-600. doi:org/10.1016/j.cell.2011.01.015 41.

Dufrêne, Y. (2015) ‘Sticky microbes: forces in microbial cell adhesion’, *Trends Microbiology*, 23(6), pp.376-382. doi:org/10.1016/j.tim.2015.01.011.

Dunson, W. and Travis, J. (1991) ‘The Role of Abiotic Factors in Community Organization’, *The American Naturalist*, 138(5), pp.1067-1091. doi:10.1086/285270.

Dworking, M. and Gutnick, D. (2017) ‘Sergei Winogradsky: a founder of modern microbiology and the first microbial ecologist’, *FEMS Microbiology Reviews*, 36(2), pp.364-379. doi:org/10.1111/j.1574-6976.2011.00299.x.

Dykhuizen, D. (1990) 'Experimental studies of Natural Selection in Bacteria' *Annual Review of Ecology and Systematics*, 21, pp.373-398. doi:org/10.1146/annurev.es.21.110190.002105.

Eames, M. and Kortemme, T. (2012) 'Cost-benefit trade-offs in Engineered *lac* Operons', *Science*, 336(6083), pp.911-915. doi:10.1126/science.1219083.

Edwards, S. and KJellerup, B. (2013) 'Applications of biofilms and biotransformation of persistent organic pollutants, pharmaceuticals/ personal care products, and heavy metals', *Applied Microbiology and Biotechnology*, 97, pp.9909-9921. doi:org/10.1007/s00253-013-5216-z.

Egunov M. (1926) 'On a plate of sulphur bacteria in the Black Sea' [In Russian] *Findings of the Odessa Agricultural Institute*, pp.49-60.

Egunov, M. (1895) 'Sulfur bacterium of Odessa estuaries', [In Russian] *Arch Biological Sciences*, *Imperial Institute of Experimental Medicine in St. Petersburg*, 3, pp 378-393.

Ehrlich, P. and Wilson, E. (1991) 'Biodiversity Studies: Science and Policy', *Science,* 253(5021), pp.758-762. doi:10.1126/science.253.5021.758.

Eisenhauer, N., Scheu, S. and Jousset, A. (2012) 'Bacterial Diversity Stabilizes Community Productivity', *PLoS ONE,* 7(3), e34517. doi:org/10.1371/journal.pone.0034517.

Elarsi, M. and Miller, R. (1999) 'Study response of a biofilm bacterial community to UV radiation', *Applied Environmental Microbiology*, 65(5), pp.2025-2031. doi:10.1128/AEM.65.5.2025-2031.1999.

Elena, S. (2002) 'Restrictions to RNA virus adaptation: an experimental approach', *Antonie Van Leeuwenhoek,* 81, pp.135-142. doi:org/10.1023/A:1020589929125.

Elias, S and Banin, E. (2012) 'Multi-species biofilms: living with friendly neighbours', *FEMS Microbiology Reviews,* 36(5), pp.990-1004. doi:org/10.1111/j.1574-6976.2012.00325.x.

Erwin, D. (2008) 'Macroevolution of ecosystem engineering, niche construction and diversity', *Trends in Ecology and Evolution*, 23(6), pp.304-310. doi:10.1016/j.tree.2008.01.013.

Estrela, S. *et al.* (2019) 'Environmentally mediated social dilemmas', *Trends in Ecology and Evolution*, 34(1), pp.6-18. doi:org/10.1016/j.tree.2018.10.004.

Estrela, S., Trisos, C. and Brown, S. (2012) 'From Metabolism to Ecology: Cross-Feeding Interactions Shape the Balance Between Polymicrobial Conflict and Mutualism', *The American Naturalist,* 180(5), pp.556-576. doi:10.1086/667887.

Estrela, S., Whitely, M. and Brown, S. (2014) 'The demographic determinants of human microbiome health', *Trends in microbiology*, 23(3), pp.134-141. doi:10.1016/j.tim.2014.11.005.

Eydallin, G*. et al*. (2014) 'The nature of laboratory domestication changes in freshly isolated *Escherichia coli* strains', *Environmental Microbiology*, 16(3), pp.813-828. doi:10.1111/1462-2920.12208.

Ferenci, T. (1996) 'Adaptation to life at micromolar nutrient levels: the regulation of *Escherichia coli* glucose transport by endoinduction and cAMP', *FEMS Microbiology Reviews*, 18(4), pp.301-317. doi:org/10.1111/j.1574-6976.1996.tb00246.x.

Ferenci, T. (2016) ‘Trade-off mechanisms shaping the diversity of bacteria’, *Trends in Microbiology,* 24(3), pp 209-223. doi:10.1016/j.tim.2015.11.009.

Ferguson, G., Bertels, F. and Rainey, P (2013) ‘Adaptive Divergence in Experimental Populations of *Pseudomonas fluorescens*. V. Insight into the Niche Specialist Fuzzy Spreader Compels Revision of the Model *Pseudomonas* Radiation’, *Genetics*, 195(4) pp.1319-35. doi:10.1534/genetics.113.154948.

Fiegna, F.*et al.* (2015) ‘Evolution of species interactions determines microbial community productivity in new environments’, *The ISME Journal*, 9, pp.1235-45. doi:org/10.1038/ismej.2014.215.

Fierer, N., Baberán, A. and Laughlin, D. (2014) ‘Seeing the forest for the genes: using metagenomics to infer the aggregated traits of microbial communities’, *Frontiers Microbiology*, 5(614). doi:org/10.3389/fmicb.2014.00614.

Filkins, L. *et al*. (2015) ‘Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* Drives *S. aureus* towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model.’, *Journal Bacteriology*, 197, pp.2252-2264. doi:10.1128/JB.00059-15.

Flemming, H. and Weurtz, S (2019) ‘Bacteria and archea on Earth and their abundance in biofilms’ *Nature Review Microbiology*, 17(4), pp.247-260. doi:10.1038/s41579-019-0158-9.

Flemming, H. and Wingender, J. (2010) ‘The Biofilm matrix’, *Nature reviews*, 8, pp.623-633. doi:10.1038/nrmicro2415.

Fletcher, M. and Pringle, J. (1985) ‘The effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces’, *Journal of Colloid and interface Science*, 104(1), pp.5-14. doi:org/10.1016/0021-9797(85)90004-9.

Flynn, K. *et al*. (2016) ‘Evolution of ecological diversity in biofilms of *Pseudomonas aeruginosa* by altered cyclic diguanylate signalling’, *Journal of Bacteriology,* 198, pp.2608-2618. doi:10.1128/JB.00048-16.

Flynn, K. *et al.* (2016) ‘Evolution of Ecological Diversity in Biofilms of *Pseudomonas aeruginosa* by Altered Cyclic diguanylate Signalling’, *Journal of Bacteriology,* 198, pp.2608-2618. doi:10.1128/JB.00048-16.

Fodor, A. *et al*. (2012) ‘The Adult Cystic Fibrosis Airway Microbiota is Stable over Time and Infection Type, and Highly Resilient to Antibiotic Treatment of Exacerbations’, *PLoS ONE,* 7(9), e45001. doi:org/10.1371/journal.pone.0045001.

Fong, J. and Yildiz, F. (2007) ‘The *rbmBCDEF* Gene Cluster Modulates Development of Rugose Colony Morphology and Biofilm Formation in *Vibrio cholerae*’, *Journal of Bacteriology,* 189, pp.2319-2330. doi:10.1128/JB.01569-06.

Fontana, J. *et al*. (1990) ‘*Acetobacter* cellulose pellicles as a temporary skin substitute’, applied biochemistry and biotechnology, 24, pp.253-264. doi:org/10.1007/BF02920250.

Fontana, S. *et al*. (2018) ‘Individual-level trait diversity predicts phytoplankton community properties better than species richness or evenness’, *The ISME Journal*, 12, pp.356-366. doi:org/10.1038/ismej.2017.160.

Foster, K. and Bell, T. (2012) ‘Competition, Not cooperation, dominates Interactions among culturable Microbial Species’, *Current Biology*, 22(19), pp.1845-1850. doi:org/10.1016/j.cub.2012.08.005.

Fotana, S., Petchey, O. and Pomati, F. (2016) ‘Individual-level trait diversity concepts and indices to comprehensively describe community change in multidimensional trait space’, *Functional Ecology*, 30(5), pp.808-81. doi:org/10.1111/1365-2435.12551.

Frank, S. (1998) *Foundations of social evolution*. Princeton New Jersey: Princeton University Press.

Franklin, M. and Ohman, D (1996) ‘Identification of algI and algJ in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which hare required for alginate O acetylation’ *Journal of Bacteriology*, 178(8), pp.2186-2195. doi:10.1128/jb.178.8.2186-2195.1996.

Friedman, J. and Gore, J. (2016) 'Ecological systems biology: The dynamics of interacting populations', *Current Opinion in Systems Biology*, 1, pp.114-121. doi:org/10.1016/j.coisb.2016.12.001.

Friesen, M. *et al*. ‘Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in *Escherichia coli*’, Evolution, 58(2), pp.245-260. doi:org/10.1111/j.0014-3820.2004.tb01642.x.

Garnier, A. *et al*. (2019) ‘Temporal scale dependent interactions between multiple environmental disturbances in microcosm ecosystems’, *Global Change Biology*, 23(12), pp.5237-5248. doi:org/10.1111/gcb.13786.

Gause, G. (1932) ‘Experimental studies on the struggle for existence’, *Journal of Experimental Biology,* 9, pp.389-402.

Gehrig, S. (2005) ‘Adaptation of *Pseudomonas fluorescens* SBW25 to the air-liquid interface: a study in evolutionary genetics’, *DPhil Thesis*. Oxford: University of Oxford.

Gillespie J. (1974) ‘Natural selection for within-generation variance in offspring numbers’, *Genetics,* 76(3), pp.601-606.

Gleason, H. (1926) ‘The Individualistic Concept of the Plant Association’, *Bull Torrey Botanical Club*, 53, pp.7-26.

Glick, B. and Patten, C. (1999) *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria.* London: Imperial College Press.

Goeres, D. *et al*. (2009) ‘A method for growing a biofilm under low shear at the air–liquid interface using the drip flow biofilm reactor’, *Nature Protocols*, 4, pp.783-788. doi:org/10.1038/nprot.2009.59.

Golby, S*. et al*. (2012) ‘Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond’, *FEMS Microbial Ecology*, 79(1), pp 240-250. doi:org/10.1111/j.1574-6941.2011.01212.x.

Goldford, J. *et al.* (2018) ‘Emergent simplicity in microbial community assembly, *Science*, 361(6401), pp.469-474. doi:10.1126/science.aat1168.

Gooderham, J. and Hancock, R. (2009) ‘Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa’*, *FEMS Microbiology Reviews*, 33(2), pp.279-294. Doi:org/10.1111/j.1574-6976.2008.00135.x.

Grant, P. (1986) Ecology and Evolution of Darwin’s Finches. Princeton New Jersey: Princeton University Press.

Green, J *et al*. (2011) ‘Evolution in a test tube: rise of the Wrinkly Spreaders’, *Journal of Biological Education*, 45(1), pp.54-59. doi:10.1080/00219266.2011.537842

Griffin, A., West, S. and Buckling, A. (2004) ‘Cooperation and competition in pathogenic bacteria’, *Nature*, 430, pp.1024-1027. doi:org/10.1038/nature02744.

Grimbergen, A. *et al.* (2015) ‘Microbial bet-hedging; the power of being different’, *Current Opinion in Microbiology*, 25, pp.67-72. doi:org/10.1016/j.mib.2015.04.008.

Großkopf, T. and Soyer, O. (2014) ‘Synthetic microbial communities’, *Current Opinion in Microbiology*, 18, pp.72-77. doi:org/10.1016/j.mib.2014.02.002.

Guanglai, L., Lick-Kong, T. and Jay X, T. (2008) ‘Amplified effect of Brownian motion in bacterial near-surface swimming’, *PNAS*, 105(47), pp.18355-18359. doi:10.1073/pnas.0807305105.

Gutiérrez, J and Jones, C. (2008) *Ecosystem engineers*. John Wiley and Sons Ltd. doi.org/10.1002/9780470015902.a0021226.

Gutiérrez, J. and Jones, C. ‘Physical ecosystem engineers as agents of biogeochemical heterogeneity’, *BioScience*, 56(3), pp.227-236. doi:org/10.1641/0006-3568(2006)056[0227:PEEAAO]2.0.CO;2.

Guttenplan, S. and Kearns, D. (2013) ‘Regulation of flagellar motility during biofilm formation’, *FEMS Microbiology Reviews,* 37(6), pp.849-871. doi:org/10.1111/1574-6976.12018.

Güvener, Z. and Harwood, C. (2007) ‘Subcellular location characteristics of the Pseudomonas aeruginosa GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces,’ *Molecular Microbiology*, 66(6), pp.1459-1473. doi:org/10.1111/j.1365-2958.2007.06008.x

Ha, D. and O’Toole, G. (2015) ‘c-di-GHP and it’s effect on biofilm formation and dispersion: a *Pseudomonas aeruginosa* review’, in Ghannoum, M. *et al*. (ed.) *Microbial Biofilms.* 2nd edn*.* Washington, DC: American Society for Microbiology. doi:10.1128/microbiolspec.MB-0003-2014.

Haines, J. and Alexander, M. (1975) ‘Microbial Degradation of Polyethylene Glycols’*, Applied Microbiology*, 29(5), pp.621-625.

Hall-Stoodley, L. and Stoodley, P. (2002) 'Developmental regulation of microbial biofilms' *Current Opinion in Biotechnology*, 13(3), pp.228-233. doi:org/10.1016/S0958-1669(02)00318-X.

Hall-Stoodley, L., Costerton, J. and Stoodley, P. (2004) ‘Bacterial biofilms: from the Natural environment to infectious diseases, *Nature Reviews Microbiology*, 2, pp.95-108. doi:org/10.1038/nrmicro821.

Hansen, S. *et al*. (2007) ‘Evolution of species interactions in a biofilm community’, *Nature,* 445 pp.533-536. doi:org/10.1038/nature05514.

Hardin, G. (1968) ‘The tragedy of the commons’, *Science,* 162 (3859), pp.1243-1248.

Harrison, F. and Buckling, A. (2009) ‘Siderophore production and biofilm formation as linked social traits’, *The ISME Journal*, 3, pp.632-634. doi:org/10.1038/ismej.2009.9.

Harrison, J. *et al*. (2010) ‘Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening’, *Nature Protocols*, 5, pp.1236-1254. doi:org/10.1038/nprot.2010.71.

Harrison, Z. *et al.* (2020) ‘Local Delivery of Anti-biofilm therapeutics’, in Li, B*. et al.* (eds) *Racing for the Surface*. Springer, Cham. pp.447-510. doi:org/10.1007/978-3-030-34475-7_21.

Hassett, D. *et al*. (2002) ‘Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment and strategies and drug targets’, *Advanced Drug Delivery Reviews*, 54(11), pp.1425-1443. doi:org/10.1016/S0169-409X(02)00152-7.

Hauer, A. (2009) ‘The type III secretion system of *Pseudomonas aeruginosa*; infection by injection, *Nature Reviews Microbiology*, 7, pp.654-665. doi:org/10.1038/nrmicro2199.

Hector, A., Griese, M. and Hartl, D. (2014) ‘Oxidative stress in cystic fibrosis lung disease: an early event, but worth targeting’, *European Respiratory Journal*, 44, pp.17-19. doi:10.1183/09031936.00038114.

Henrici, A. (1933) ‘Studies of freshwater bacteria: I. A direct microscopic technique’, *Journal of Bacteriology,* 25(3), pp.277-87.

Hibbing, M. *et al*. (2010) ‘Bacterial competition: surviving and thriving in the microbial jungle’, *Nature Reviews Microbiology*, 8, pp.15-25. doi:org/10.1038/nrmicro2259.

Hickmann, J., Tifream, D. and Harwoodm, C. (2005) ‘A chemosensory system that regulates biofilm through modulation of cyclic diguanylate levels’, *PNAS*, 102(4), pp.14422-14427. doi:org/10.1073/pnas.0507170102.

Hill, N. and Pedley, T. (2005) ‘Bioconvection’, *Fluid Dynamics Research*, 37(1-2), pp.1-20. doi:10.1016/j.fluiddyn.2005.03.002.

Hill, T. *et al.* (2003) ‘Using ecological diversity measures with bacterial communities’, *FEMS Microbiology Ecology*, 43(1), pp.1-11. doi:org/10.1111/j.1574-6941.2003.tb01040.x.

Hillebrand, H. and Matthiessen, B. (2009) ‘Biodiversity in a complex world: consolidation and progress in functional biodiversity research’, *Ecology letters*, 12, pp.1405-1419. doi:org/10.1111/j.1461-0248.2009.01388.x.

Hofinger, M., Bertholdt, G. and Weuster-Botz, D. (2011) ‘Microbial production of homogenously layered cellulose pellicles in a membrane bioreactor’, *Biotechnology and Bioengineering*, 108(9), pp.2237-2240. doi:org/10.1002/bit.23162.

Høiby, N. *et al*. (1977) ‘*Pseudomonas aeruginosa* Infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* pre-cipitins determined by means of crossed immunoelectrophoresis’, *Scandinavian Journal of Respiratory Disease*, 58(2), pp.1-96.

Hölscher ,T. *et al.* (2015) "Motility, Chemotaxis and Aerotaxis Contribute to Competitiveness During Bacterial Pellicle Biofilm Development", *Journal of Molecular Biology*, 247, pp.3695-3708. doi:10.1016/j.jmb.2015.06.014.

Holyoak, M., Leibold, M. and Holt, R. (2005) *Metacommunities: Spatial Dynamics and Ecological Communities.* Chicago: University of Chicago Press.

Howard, M., Bell, T. and Kao-Kniffin J. (2017) ‘Soil microbiome transfer method affects microbiome composition, including dominant microorganisms, in a novel environment’, *FEMS Microbiology Letters*, 364(11). doi:org/10.1093/femsle/fnx092.

Hu, X. *et al.* (2007) ‘Isolation of bacteria able to grow on both polyethylene glycol (PEG) and polypropylene glycol (PPG) and their PEG/PPG dehydrogenases’, *Applied Microbiology Biotechnology,* 73, pp.1407-1413. doi:10.1007/s00253-006-0616-y.

Huang, X. and Lin, Y. (2020) ‘Reconstruction and analysis of a three-compartment genome-scale metabolic model for *Pseudomonas fluorescens’*, *Biotechnology and Applied biochemistry,* 67(1), pp.133-139. doi:org/10.1002/bab.1852.

Hunter, J. and Cigna, A. (1981) ‘Bacterial Blight Incited in Parsnip by *Pseudomonas marginalis* and *Pseudomonas viridiflava*’, *Phytopathology*, 71, pp.1238-1241.

Huxley, J. (1942) *Evolution. The Modern Synthesis.* London: George Alien & Unwin Ltd.

Ibáñez, A., Zafra, O. and González-Pastor, J. (2017) ‘Mechanisms and Regulations of Extracellular DNA Release and Its Biological Roles in Microbial Communities’, *Frontiers in Microbiology*, 8(1390), doi:org/10.3389/fmicb.2017.01390

Jackson, C. (2003) 'Changes in Community Properties during Microbial succession', *Nordic Society Oikos*, 101(2), pp.444-448.

Jackson, C., Churchill, P. and Roden, E. (2001) 'Successional Changes in Bacterial Assemblage structure during epilithic biofilm development' *Ecology*, 82(2), pp.555-556. doi:org/10.1890/0012-9658(2001)082[0555:SCIBAS]2.0.CO;2.

Jahn, A., Griebe, T. and Nielsen, P. (1999) ‘Composition of *Pseudomonas putida* biofilms; Accumulation of protein in the biofilm matrix’, *BioFouling*, 14(1), pp.49-57. doi:org/10.1080/08927019909378396.

Jain, D. *et al.* (1991) ‘A drop-collapsing test for screening surfactant-producing microorganisms’, *Journal of Microbiological Methods*, 13(4), pp.271-279. doi:org/10.1016/0167-7012(91)90064-W.

Jánosi, I. *et al*. (2002) 'Is bioconvection enhancing bacterial growth in quiescent environments?' *Environmental Microbiology,* 4(9), pp.525-531. doi:10.1046/j.1462-2920.2002.00328.x.

Jarvik, T. *et al*. (2010) ‘Short-Term Signatures of Evolutionary Change in the *Salmonella enterica* Serovar Typhimurium 14028 genome’, *Journal of Bacteriology*, 192, pp.560-567. doi:10.1128/JB.01233-09.

Jendresen, M. and Glantz, P. (2009) ‘Clinical adhesiveness of selected dental material. An *in-vivo* study’, Acta Odontologica Scandinavica, 39(1), pp.39-45. doi:org/10.3109/00016358109162257.

Jessup, C*. et al.* (2004) ‘Big questions, small worlds: microbial model systems in ecology’, *Trends in Ecology & Evolution,*19, pp.189-197. doi:org/10.1016/j.tree.2004.01.008.

Jiang, L. and Morin, P. (2004) ‘Productivity gradients cause positive diversity-invasibility relationships in microbial communities’, *Ecology Letters*, 7, pp.1047-1057. doi:org/10.1111/j.1461-0248.2004.00660.x.

Jin, Y. *et al.* (2018) ‘Effect and mechanisms of microbial remediation of heavy metals in soil: critical review’, *Applied sciences*, 8(8), 1336. doi:org/10.3390/app8081336.

Johansen, R. *et al*. (2019) ‘Tracking Replicate Divergence in Microbial Community Composition and Function in Experimental Microcosms’, *Microbial Ecology*, 78, pp.1035-1039. doi:org/10.1007/s00248-019-01368-w.

Jones, C., Lawton, J. and Shachak, M. (1994) ‘Organisms as ecosystem engineers’ *Ecosystem Management*, 69, pp.373-386. doi:org/10.1007/978-1-4612-4018-1_14.

Jones, C., Lawton, J. and Shachak, M. (1997) ‘Positive and negative effects of organisms as ecosystem engineers’ *Ecology,* 78(7), pp.1946-1957. doi:org/10.1890/0012-9658(1997)078[1946:PANEOO]2.0.CO;2.

Jones, R. *et al*. (2009) ‘A comprehensive survey of soil acid bacterial diversity using pyrosequencing and clone library analyses’, *The ISME Journal*, 3, pp.442-453. doi:org/10.1038/ismej.2008.127.

Jong, I., Haccou, P. and Kuipers, O. (2011) ‘Bet hedging or not? A guide to proper classification of microbial survival strategies’, *BioEssays*, 33(3) pp.215-23. doi:10.1002/bies.201000127.

Josenhans, C. and Suerbaum, S. (2002) ‘The role of motility as a virulence factor in bacteria’, *International Journal of Medical Microbiology*, 291(8), pp.605-614. doi:10.1078/1438-4221-00173.

Kaltz, O. *et al.* (2012) ‘Bacterial microcosms obey Taylor’s law: effects of abiotic and biotic stress and genetics on mean and variance of population density’, *Ecological Processes,* 1(5). doi:org/10.1186/2192-1709-1-5.

Karve, S*. et al*. (2015) ‘*Escherichia coli* populations in unpredictably fluctuating environments evolve to face novel stresses through enhanced efflux activity’, *Journal of Evolutionary Biology,* 28(5), pp.1131-1143. doi:org/10.1111/jeb.12640.

Kawecki, T. *et al*. (2012) 'Experimental Evolution’, *Trends in Ecology and Evolution*, 27(10). pp.547-60. doi:org/10.1016/j.tree.2012.06.001.

Kelman, A. and Hruschka, J. (1973) ‘The role of Motility and Aerotaxis in the Selective Increase of Avirulent Bacteria in Still Broth Cultures of *Pseudomonas solanacearum,*’ *Microbiology*, 76(1). pp.177-188. doi.org/10.1099/00221287-76-1-177.

Kielwein, G. (1969) ‘Nutrient medium for the selective cultivation of pseudomonads and aeromonads’, *Archiv fur Lebensmittelhygiene*, 20, pp.131-133.

Kiesewalter, H. *et al*. (2020) ‘Secondary metabolites of *Bacillus subtilis* impact the assembly of soil-derived semisynthetic bacterial communities’, *Journal of Organic Chemistry*, 16, pp.2983-2998. doi:10.3762/bjoc.16.248.

King, E., Ward, K. and Raney, D. (1954) ‘Two simple media for the demonstration of pyocyanin and fluorescin’ *Journal of Laboratory and Clinical Medicine,* 44(2), pp.301-307. doi:org/10.5555/uri:pii:002221435490222X.

Kiørboe, T., Visser, A. and Andersen, K. (2018) ‘A trait-based approach to ocean ecology’ *ICES Journal of Marine Science*, 75(6), pp.1849-1863. doi.org/10.1093/icesjms/fsy090.

Kjelleberg, S. (1985) 'Mechanisms of bacterial adhesion at gas-liquid interfaces', in Fletcher, M and Savage, D (edt) *Bacterial adhesion: Mechanisms and Physiological Significance*. New York: Plenum Press. pp.163-194.

Kobayashi, K. (2007) '*Bacillus Subtilis* pellicle Formation Proceeds through Genetically Defined Morphological Changes', *Journal of bacteriology*, 189, pp.4920-4931. doi:10.1128/JB.00157-07.

Konopka, A. (2009) 'What is microbial community ecology', *The ISME Journal*, 3, pp.1223-1230. doi:org/10.1038/ismej.2009.88.

Koskella. B. and Brockhurst, M. (2014) 'Bacteria-phage as a driver of ecological and evolutionary processes in microbial communities', *FEMS Microbiology Reviews*, 38(5), pp.916-931. doi:org/10.1111/1574-6976.12072.

Koza, A. *et al*. (2009) 'Characterization of a novel air-liquid interface biofilm of *Pseudomonas fluorescens* SBW25', *Microbiology*, 155(5), pp1397-1406. doi:10.1099/mic.0.025064-0.

Koza, A. *et al*. (2011) 'Environmental modification and niche construction: developing O_2 gradients drive the evolution of the Wrinkly Spreader', *The ISME Journal*, 5 (4), pp.665-673. doi:10.1038/ismej.2010.156.

Koza, A. *et al*. (2017) 'Adaptive radiation of *Pseudomonas fluorescens* SBW25 in experimental microcosms provides an understanding of the evolutionary ecology and molecular biology of A-L interface biofilm formation', *FEMS Microbiology Letters*, 364(12). doi:org/10.1093/femsle/fnx109.

Kraemer, S., Toups, M. and Velicer, G. (2010) 'Natural variation in developmental life history traits of the bacterium *Myxococcus xanthus*', *FEMS Microbiology Ecology*, 73(2), pp.226-233. doi:org/10.1111/j.1574-6941.2010.00888.x.

Kramer, J., Özkaya, Ö. and Kümmerli, R. (2020) 'Bacterial siderophores in community and host interactions', *Nature Reviews Microbiology*, 18(3), pp.152-163. doi:10.1038/s41579-019-0284-4.

Krause, S. *et al.* (2014) 'Trait-based approaches for understanding microbial biodiversity and ecosystem functioning'*, Frontiers in Microbiology*, 5(251). doi.org/10.3389/fmicb.2014.00251

Krzysciak, P. *et al*. (2017) '*Acinetobacter baumannii* isolated from hospital-acquired infection: biofilm production and drug susceptibility', *Journal of Pathology, Microbiology and Immunology*, 125(11), pp.1017-1026. doi:org/10.1111/apm.12739.

Kůdela, V., Krejzar, V. and Pánková, I. (2010) '*Pseudomonas corrugate* and *Pseudomonas marginalis* Associated with the Collapse of Tomato Plants in Rockwool Slab Hydroponic Culture', *Plant protection Science*, 16 (1), pp.1-11.

Kümmerli, R. (2015) 'Cheat invasion causes bacterial trait loss in lung infections', *PNAS*, 112(34), pp.10577-10578. doi:org/10.1073/pnas.1513797112.

Kuśmierska, A. and Spiers, A. (2016) 'New insights into the effects of several environmental parameters on the relative fitness of a numerically dominant class of evolved niche specialist', *International Journal of Evolutionary Biology,* Article ID 486565. doi:org/10.1155/2016/4846565.

Laland, K., Boogert, N. and Evans, C. (2014) ‘Niche construction, innovation and complexity’, *Environmental Innovation and Societal Transitions*, 11, pp.71-86. doi:org/10.1016/j.eist.2013.08.003.

Laland, K., Matthews, B. and Feldman, M. (2016) ‘An introduction to niche construction theory’, *Evolutionary Ecology*, 30, pp.191-202. doi:10.1007/s10682-016-9821-z.

Lanfear, R., Kokko, H. and Eyre-Walker, A. (2014) ‘Population size and the rate of evolution’, *Trends in Ecology and Evolution*, 29, pp.33-41. doi:org/10.1016/j.tree .2013.09.009.

Lau, G. *et al*. (2004) ‘The role of pyocyanin in *Pseudomonas aeruginosa* infection’, *Trends in molecular medicine*, 10(12), pp.599-606. doi:org/10.1016/j.molmed.2004.10.002.

Laue, H. *et al.* (2006) ‘Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*’, *Microbiology*, 152(10), pp.2909-2918. doi:org/10.1099/mic.0.28875-0.

Lawrence, D. *et al*. (2012) ‘Species interactions alter evolutionary responses to a novel environment’, *PLoS Biology*, 10(5), e1001330. doi:org/10.1371/journal.pbio.1001330.

Lawrence, J. *et al.* (1991) ‘Optical sectioning of microbial biofilms’, *Journal of Bacteriology*, 173, pp.6558-6567. doi:10.1128/jb.173.20.6558-6567.1991.

Lawton, J. (1999) ‘Are There General Laws in Ecology?’, *Oikos*, 84(2), pp.177-192. doi:org/10.2307/3546712.

Le Quéré, B. and Ghigo, J. (2009) ‘BcsQ is an essential component of the Escherichia coli cellulose biosynthesis apparatus that localizes at the bacterial cell pole’, *Molecular Microbiology*, 72(3), pp.724-740. doi:org/10.1111/j.1365-2958.2009.06678.x.

Lee, W., van Baalen, M. and Jansen, V. (2016) ‘Siderophore production and the evolution of investment in a public good; An adaptive dynamics approach to kin selection’, *Journal of Theoretical Biology*, 388, pp.61-71. doi:org/10.1016/j.jtbi.2015.09.038.

Leiby, N., Harcombe, W. and Marx, C. (2012) ‘Multiple long-term, experimentally-evolved populations of *Escherichia coli* acquire dependence upon citrate as an iron chelator for optimal growth on glucose’, *BMC Evolutionary Biology,* 12(151). doi:org/10.1186/1471-2148-12-151.

Leisinger, T. and Margraff, R. (1979) ‘Secondary metabolites of the fluorescent pseudomonads’, *Microbiology Reviews,* 43(3), pp.422-422.

Lenski, R. (2017) ‘Experimental evolution and the dynamics of adaption and genome evolution in microbial populations’, *The ISME Journal*, 11, pp.2128-2194.

Lenski, R. and Mongold, J. (2000) ‘Cell size, shape, and fitness in evolving populations of bacteria’, in Brown, J and West, G. (ed.) *Scaling in biology*. Oxford, UK: Oxford University Press. pp.221-235.

Lenski, R. and Travisano, M. (1994) ‘Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations’, *PNAS*, 91(15), pp.6808-6814. doi:org/10.1073/pnas.91.15.6808.

Lenski, R. *et al*. (1991) ‘Long-term experimental evolution in *Escherichia coli.* I. Adaption and divergence during 2000 generations’, *The American Naturalist*, 138(6), pp.1315-1341. doi:10.1086/285289.

Lewontin, R. (1974). *The genetic basis of evolutionary change*. New York, NY: Columbia University Press.

Li, S. *et al.* 'Niche and fitness differences determine invasion success and impact in laboratory bacterial communities' *The ISME Journal*, 13(2), pp.402-412. doi.org/10.1038/s41396-018-0283-x.

Liang, Y. *et al*. 'Pellicle formation in *Shewanella oneidensis*' *BMC Microbiology*, 10, 291. doi:org/10.1186/1471-2180-10-291.

Lim, J., May, J. and Cegelski, L. (2012) 'Dimethyl sulfoxide and ethanol elicit increased amyloid biogenesis and amyloid-integrated biofilm formation in *Escherichia coli*', *Applied and Environmental Microbiology,* 78, pp.3369-3378. doi:10.1128/AEM.07743-11.

Lind, A., Farr, A. and Rainey, P. (2017) 'Evolutionary convergence in experimental *Pseudomonas* populations', *The ISME Journal*, 11, pp.589-600. doi:org/10.1038/ismej.2016.157.

Lind, P., Farr, A. and Rainey, P. (2015) 'Experimental evolution reveals hidden diversity in evolutionary pathways', *eLife*, 4, e07074. doi:10.7554/eLife.07074.

Loper, L. *et al*. (2012) 'Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions', *PLoS Genetics*, 8, e1002784. doi:org/10.1371/journal.pgen.1002784.

Lopes, S*. et al*. (2012) 'Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection', *International Journal of Antimicrobial Agents*, 40(3), pp.260-263. doi:org/10.1016/j.ijantimicag.2012.04.020.

Losos, J. and Mahler, D. (2010) 'Adaptive radiation: The interaction of ecological opportunity, adaptation, and speciation' in Bell, M. *et al*. (ed.) *Evolution Since Darwin: The First 150 Years*. Sunderland, USA: Sinauer Associates. pp.381-420.

Lugtenberg, B. and Kamilova, F. (2009) 'Plant-growth-promoting rhizobacteria', *Annual Review of Microbiology*, 63, pp.541-556. doi:org/10.1146/annurev.micro.62.081307.162918.

Lynch, M. (2007) 'The frailty of adaptive hypotheses for the origins of organismal complexity', *PNAS*, 104, pp.8597-8604. doi:org/10.1073/pnas.0702207104.

MacLaughlin, Y. (2016) MbR Thesis. Dundee: Abertay University.

Maclean, R. (2005) 'Adaptive radiation in microbial microcosms', *Journal of Evolutionary Biology*, 18(6), pp.1376-1386.doi:org/10.1111/j.1420-9101.2005.00931.x.

Maclean, R. (2008) 'The tragedy of the commons in microbial populations: insights from theoretical, comparative and experimental studies', *Heredity*, 100, pp.471-477. doi:org/10.1038/sj.hdy.6801073x.

Maclean, R. and Bell, G. (2003) 'Divergent evolution during an experimental adaptive radiation', *Proceedings of The Royal Society B*, 270(1524). doi:org/10.1098/rspb.2003.2408.

Maclean, R. and Bell, G. and Rainey, P. (2004) 'The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens'*, *PNAS*, 101(21), pp.8072-8077. doi:org/10.1073/pnas.0307195101.

MacLean, R., Dickson, A. and Bell, G. (2005) 'Resource competition and adaptive radiation in a microbial microcosm', *Ecology Letter*, 8(1), pp.38-46. doi:org/10.1111/j.1461-0248.2004.00689.x.

Madsen, J. *et al.* (2015) ‘Facultative Control of Matrix Production Optimizes Competitive Fitness in *Pseudomonas aeruginosa* PA14 Biofilm Models’, *Applied and Environmental Microbiology*, 81(21), pp.8414-8426. doi:10.1128/AEM.02628-15.

Magalhães, A. *et al.* (2016) ‘The cystic fibrosis microbiome I an ecological perspective and its impact in antibiotic therapy’, *Applied Microbiology and Biotechnology*, 100, pp.1163-1181. doi:org/10.1007/s00253-015-7177-x.

Mallick, H. *et al*. (2019) ‘Predictive metabolomic profiling of microbial communities using amplicon or metagenomic sequencing’ *Nature Communications*, 10, 3136. doi:org/10.1038/s41467-019-10927-1.

Mandakhalikar, K. *et al.* (2018) ‘Extraction and quantification of biofilm bacteria: Method optimised for urinary catheters’ *Scientific reports*, 8(8069). doi:10.1038/s41598-018-26342-3.

Marsh, P. (2018) ‘In sickness and in health - what does the oral microbiome meant for us? An ecological perspective’, *Advances in Dental research*, 29(1), pp.60-65. doi:org/10.1177/0022034517735295.

Marshall, H. and Burchardt, L. (2005) 'Neuston: its definition with a historical review regarding its concept and community structure', *Archives for Hydrobiology*, 164(4), pp.429-448. doi:10.1127/0003-9136/2005/0164-0429.

Martz, C. *et al*. (2005) 'Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholera*' *PNAS*, 102(46), pp.16819-16824. doi:org/10.1073/pnas.0505350102.

Massol-Deyá, A. *et al.* (1995) ‘Channel structure in aerobic biofilms of fixed film reactors treating contaminated groundwater’, *Applied and Environmental Microbiology*, 61(2), pp.769-777.

Matthews, B. *et al*. (2014) ‘Under niche construction: an operational bridge between ecology, evolution and ecosystem science’, *Ecological Monographs*, 84(2), pp.245-263. doi:org/10.1890/13-0953.1.

Mayr, E. (1942) *Systematics and the origins of species*. New York: Columbia university press.

McDonald, M. *et al.* (2011) ‘The distribution of fitness effects of new beneficial mutations in *Pseudomonas fluorescens*’, *Biology Letters*, 7(1), pp.98-100. doi:org/10.1098/rsbl.2010.0547.

McDonald, S. *et al*. (2009) “Adaptive divergence in experimental populations of Pseudomonas fluorescens. IV. Genetic constraints guide evolutionary trajectories in a parallel adaptive radiation,” *Genetics*, 183(3), pp.1041-1053. doi:org/10.1534/genetics.109.107110.

McGill, B. (2006) ‘Rebuilding community ecology from functional traits’, *Trends in Ecology and Evolution*, 21(4), pp.178-185. doi:org/10.1016/j.tree.2006.02.002.

McNally, L. and Brown, S. (2015) ‘Building the microbiome in health and disease: niche construction and social conflict in bacteria’, *Philosophical Transactions of the Royal Society B*. doi:org/10.1098/rstb.2014.0298.

Meier, J., Piva. A. and Fortin, D. (2012) ‘Enrichment of sulfate-reducing bacteria and resulting mineral formation in media mimicking pore water metal ion concentrations an pH

conditions of acidic pit lakes', *FEMS Microbiology Ecology*, 79(1), pp.69-84. doi.org/10.1111/j.1574-6941.2011.01199.x.

Meyer, J. *et al*. (1998) 'Siderotpying of fluorescent pseudomonads: characterization of pyoverdines of *Pseudomonas fluorescens* and *Pseudomonas putida* strain from Antartica', *Microbiology*, 144(11), pp.3119-3126.

Migula, W. (1894) 'Über ein neues System der Bakterien', *Arb Bakteriol Inst Karlsruhe*, 1, pp.235-328.

Miller, J. (1972) *Experiments in molecular Genetics*. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory.

Mitchell, J. and Kogure, K. (2006) 'Bacterial motility: links to the environment and a driving force for microbial physics', *FEMS Microbiology Ecology*, 55(1), pp.3-16. doi:10.1111/j.1574-6941.2005.00003.

Mittelbach, G. *et al*. (2001) 'What is the relationship between species richness and productivity?', *Ecology*, 82(9), pp.2381-2396. doi:org/10.1890/0012-9658(2001)082[2381:WITORB]2.0.CO;2.

Monds, R. and O''Toole, G. (2009) 'The developmental model of microbial biofilms; ten years of a paradigm up for review', *Trends in Microbiology*, 17(2), pp.73-87. doi:org/10.1016/j.tim.2008.11.001.

Moon, C. *et al*. (2008) 'Genomic, genetic and structural analysis of pyoverdine-mediated iron acquisition in the plan growth promoting bacterium *Pseudomonas fluorescens* SBW25', *BMC Microbiology*, 8(7), doi:org/10.1186/1471-2180-8-7.

Moonmangmee, S. *et al*. (2002a) A novel polysaccharide involved in the pellicle formation of *Acetobacter aceti*', *Journal of Bioscience Bioengineering,* 93, pp.192-200. doi:org/10.1016/S1389-1723(02)80013-5.

Morris, C. and Monier, J. (2003) 'The ecological significance of biofilm formation by plant-associated bacteria', *Annual Reviews of Phypathology*, 41, pp.429-453. doi:org/10.1146/annurev.phyto.41.022103.134521.

Moshynets, O., Boretska, M. and Spiers, A. (2013) 'Form Winodrasky column to Contemporary research using bacterial microcosms', in Harris, C. (edt). *Microcosms*. Nova science Publishers, Inc.

Myers, J., Curtis, B. and Curtis, W. (2013). 'Improving accuracy of cell and chromophore concentration measurements using optical density', *BMC Biophysics,* 6(4).

Nadell, C. and Bassler, B (2011) 'A fitness trade-off between local competition and dispersal in *Vibrio cholerae* biofilms', *PNAS*, 108(34), pp.14181-14185. doi:10.1073/pnas.1111147108.

Nadell, C., Xavier, J. and Foster, K. (2009) 'The sociobiology of biofilms.' *FEMS Microbiology Reviews*, 33(1), pp.206-224. doi:org/10.1111/j.1574-6976.2008.00150.x.

Naeem, S., Kawabata, Z. and Loreau, M. (1998). 'Transcending boundaries in biodiversity research', *Trends in Ecology and Evolution*, 13(4), pp.134-135. doi:10.1016/s0169-5347(97)01316-5.

National Institutes of Health (2002) *Research on Microbial biofilms* (PA-03-047). Available at Http;;/grants.nih.gov/grnats/guides/PA-files/PA-03-047.html (Accessed: 7 March, 2020).

Naves, P. *et al.* (2008) ‘Correlation between virulence factors and in vitro biofilm formation by *Escherichia coli* strains’, *Microbial pathogenesis*, 46(2), pp.86-91. doi:org/10.1016/j.micpath.2008.03.003.

Ndoye, B. *et al*. (2011) ‘A review of the molecular approaches to investigate the activity of cheese microbiota’, *Dairy Science and Technology*, 91, pp.495-524. doi:10.1007/s13594-011-0031-8.

Neilands, J. (1981) ‘Iron absorption and transport in microorganisms’, *Annual Review of Nutrition*, 1, pp.27-46. doi:org/10.1146/annurev.nu.01.070181.000331.

Nelson, K. *et al.* (2002). ‘Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440’, *Environmental Microbiology,* 4(12), pp.799-808. doi:org/10.1046/j.1462-2920.2002.00366.

Nemergut, D. *et al.* (2013) ‘Patterns and Processes of Microbial Community Assembly’, *Microbiology and Molecular Biology Reviews*, 77(3). pp.342-356. doi:10.1128/MMBR.00051-12

Neu, T. and Lawrence, J. (2015) ‘Innovative techniques, sensors, and approaches for imaging biofilms at different scales’, *Trends in Microbiology*, 23(4), pp.233-242. doi:org/10.1016/j.tim.2014.12.010.

Nivens, D. *et al*. (2001) 'Role of Alginate and Its O acetylation in Formation of *Pseudomonas aeruginosa* Microcolonies and Biofilms’, *Journal of Bacteriology*, 183 (3), pp.1047-1057. doi:10.1128/JB.183.3.1047-1057.2001.

Noll, M. *et al*. (2005) ‘Succession of bacterial community structure and diversity in a paddy soil oxygen gradient’, *Environmental Microbiology*, 7(3), pp.382-395. doi:org/10.1111/j.1462-2920.2005.00700.x.

Obradors, N. and Aguilar, J. (1991) ‘Efficient biodegradation of high-molecular weight polyethylene glycols by pure cultures of Pseudomonas Stutzeri’, Applied and Environmental Microbiology, 57(8), pp.2383-2388. doi:10.1128/AEM.57.8.2383-2388.1991.

Oliver, A. et al. (2000) ‘High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection’, Science, 288(5469), pp.1251-1254. doi:10.1126/science.288.5469.1251.

O'Malley, M. (2018) 'The Experimental Study of Bacterial Evolution and Its Implications for the Modern Synthesis of Evolutionary Biology', History of Biology, 51, pp.319-354. doi:org/10.1007/s10739-017-9493-8.

O'Toole, G., Kaplan, H. and Kotler, R. (2000). 'Biofilm formation as microbial development', Annual Review Microbiology, 54, pp.49-79. doi:org/10.1146/annurev.micro.54.1.49.

Ou, C. et al. (2020) ‘Prevalence of multidrug-resistant Staphylococcus aureus isolates with strong biofilm formation ability among animal-based food in shanghai’, Food control, 112. doi:org/10.1016/j.foodcont.2020.107106.

Paerl, H. and Priscu, J. (1998) ‘Microbial Phototrophic, Heterotrophic, and Diazotrophic Activities Associated with Aggregates in the Permanent Ice Cover of Lake Bonney, Antarctica’, Microbiol Ecology, 36, pp.221-230. doi:org/10.1007/s002489900109.

Palleroni, N. (1973) ‘Nucleic acid homologies in the genus Pseudomonas’, *International Journal of Systematic and Evolutionary Microbiology*, 23(4), pp.333-339. doi:org/10.1099/00207713-23-4-333.

Palleroni, N. (2008) ‘The road to the taxonomy of *Pseudomonas*’, in Cornelis, P. (ed.) *Pseudomonas: Genomics and Molecular Biology.* Norfolk, UK: Caister Academic Press, pp.1-18.

Palleroni, N. (2010) ‘The *Pseudomonas* Story’, *Environmental Microbiology*, 12(6), pp.1377-1383. doi:org/10.1111/j.1462-2920.2009.02041.

Palleroni, N. (2015) ‘*Pseudomonas*’, in Bergey, D. *et al*. (ed). *Bergey's Manual of Systematics of Archaea and Bacteria.* John Wiley & Sons. pp.1-105. doi:10.1002/9781118960608.gbm01210.

Palmer, M. (1994) ‘Variation in species richness: Towards a unification of hypotheses’, *Folia Geobotanica et Phytotaxonomica*, 29(511), pp.511–530. doi:org/10.1007/BF02883148.

Pamp, S., Sternberg, C. and Tolker-Nielsen, T. (2009) ‘Insight into the microbial multicellular lifestyle via flow-cell technology and confocal microscopy’, *Cytometry Part A,* 75A(2), pp.90-103. doi:org/10.1002/cyto.a.20685.

Pande, S. *et al.* (2014) ‘Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria’, *The ISME Journal*, 8, pp.953-962. doi:org/10.1038/ismej.2013.211.

Parsek, M. and Greenberg, E. (2005) ‘Sociomicrobiology: the connections between quorum sensing and biofilms’, *Trends in Microbiology*, 13(1), pp.27-33. doi:org/10.1016/j.tim.2004.11.007.

Parsek, M. and Singh P. (2003) ‘Bacterial biofilms: an emerging link to disease pathogenesis’, *Annual Reviews in Microbiology*, 57, pp.677-701. doi:org/10.1146/annurev.micro.57.030502.090720.

Pastar, I. *et al*. (2013) ‘Interactions of methicillin resistant Staphylococcus aureus USA300 and *Pseudomonas aeruginosa* in polymicrobial wound infection’, *PLoS ONE*, 8(2), e56846. doi:org/10.1371/journal.pone.0056846.

Pasteur, L. (1864) ‘Memoire sur la fermentation acetique’, Annales Scientifiques de l’École Normale Supériure, pp.133-158.

Paulsen, I. *et al.* (2005) ‘Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5’, *Nature Biotechnology*, 23, pp.873-878. doi:org/10.1038/nbt1110.

Pedley, T. and Kessler, J, (1992) 'Hydrodynamic Phenomena in Suspensions of Swimming Microorganisms', *Annual Reviews Fluid Mechanics*, 24, pp.313-358. doi:10.1146/annurev.fl.24.010192.001525.

Pelletier, F., Garant, D. and Hendry, A. (2009) ‘Eco-evolutionary dynamics’, *Philosophical Transactions of the Royal Society B*, 364(1523), pp.1483-1489. doi:org/10.1098/rstb.2009.0027.

Pigliucci, M. (2001) *Phenotypic Plasticity: beyond nature and nurture*. Baltimore: The John Hopkins University Press.

Ping, L., Birkenbeil, J. and Monajembashi, S. (2013) ‘Swimming behaviour of the monotrichous bacterium *Pseudomonas fluorescens* SBW25’, *FEMS Microbiology Ecology,* 86(1), pp.36-44. doi:10.1111/1574-6941.12076.

Pinu, F. and Villas-Boas, S. (2016) ‘Extracellular Microbial Metabolomics: The state of the art’ *Metabolites*, 7(3), 43. doi:org/10.3390/metabo7030043.

Poltak, S. and Cooper, V. (2010) ‘Ecological succession in long-term experimentally evolved biofilms produce synergistic communities’, *The ISME Journal*, 5, pp.369-378. doi:org/10.1038/ismej.2010.136.

Popat, R. *et al*. (2012) ‘Quorum-sensing and cheating in bacterial biofilms’, *Proceedings of the Royal Society B*, 279(1748), pp.4765-4771. doi:org/10.1098/rspb.2012.1976.

Porter, S and Rice, K. (2013) ‘Trade-offs, spatial heterogeneity, and the maintenance of microbial diversity’, *Evolution*, 67(2), pp.599-608. doi:org/10.1111/j.1558-5646.2012.01788.x.

Potvin, E., Sanschagrin, F. and Levesque, R. (2008) ‘Sigma factors in Pseudomonas aeruginosa’, *FEMS Microbiology Reviews*, 32(1), pp.38-55. doi:org/10.1111/j.1574-6976.2007.00092.x.

Preston, G., Bertrand, N. and Rainey, P. (2001) ‘Type III secretion in plant growth-promoting *Pseudomonas fluorescens* SBW25’, *Molecular Microbiology*, 41(5), pp.999-1014. doi:org/10.1046/j.1365-2958.2001.02560.x.

Queiroc, K. (2005) ‘Ernst Mayr and the modern concept of species’, *PNAS*, 102, pp.6600-6607. doi:org/10.1073/pnas.0502030102.

Rabalais, N. and Nixon, S. (2002) ‘Nutrient over-enrichment of the coastal Zone’, *Estuaries and Coasts*, 25(4). doi:10.1007/BF02804896.

Rainey, P. and Bailey, M. (1996) 'Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome' *Molecular Microbiology*, 19(3), pp.521-533. doi:org/10.1046/j.1365-2958.1996.391926.x.

Rainey, P. and Rainey, K. (2003) ‘Evolution of cooperation and conflict in experimental bacterial populations’, *Nature*, 425, pp.72-74. doi:org/10.1038/nature01906.

Rainey, P. and Travisano, M. (1998) 'Adaptive radiation in a heterogenous environment', *Nature*, 394(6688), pp.69-72.

Ramana, K., Tomar, A. and Singh, L. (2000) ‘Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*’, *World Journal of Microbiology and Biotechnology*, 16, pp.245-248. doi:org/10.1023/A:1008958014270.

Ratzke, C. and Gore, J. (2018) ‘Modifying and reacting to the environmental pH can drive bacterial interactions’, *PLOS Biology*, 16(3), e2004248. doi:org/10.1371/journal.pbio.2004248

Reiss, J. *et al.* (2009) ‘Emerging horizons in biodiversity and ecosystem functioning research’, *Trends in Ecology and Evolution*, 24(9), pp.505-514. doi:org/10.1016/j.tree.2009.03.018.

Ren, D. *et al*. (2015) ‘High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation’, *The ISME Journal*, 9, pp.81-89. doi:org/10.1038/ismej.2014.96.

Richter, A. *et al*. (2018) ‘Hampered motility promotes the evolution of wrinkly phenotype in *Bacillus subtilis*’, *BMC Evolutionary Biology*, 18(155). doi:org/10.1186/s12862-018-1266-2.

Riley, M. and Wertz, J. (2002) ‘Bacteriocins: Evolution, Ecology and Application’, *Annual Review of Microbiology*, 56, pp.117-137. doi:org/10.1146/annurev.micro.56.012302.161024.

Røder, H. *et al*. (2015) ‘Interspecies interactions result in enhanced biofilm formation by co-cultures of bacteria isolated from a food processing environment’, *Food Microbiology*, 51, pp. 18-24. doi:org/10.1016/j.fm.2015.04.008.

Røder, H. *et al.* (2020) ‘Unravelling interspecies interactions across heterogeneities in complex biofilm communities’, *Environmental Microbiology*, 22(1), pp.5-16. doi:org/10.1111/1462-2920.14834.

Røder, H., Sørensen, S. and Burmølle, M. (2016) ‘Studying Bacterial multispecies biofilms: where to start?’, *Trends in Microbiology,* 24(6), pp.503-513. doi:org/10.1016/j.tim.2016.02.019.

Romero, D. *et al*. (2011) ‘Antibiotics as Signal Molecules’, *Chemical Reviews*, 111(9), pp.5492-5505. doi:org/10.1021/cr2000509.

Römling, U., Galperin, M. and Gomelsky, M. (2013) ‘Cyclic di-GMP: The First 25 years of a Universal Bacterial Second Messenger’, Microbiology and Molecular Biology Reviews, 77(1), pp.1-52. doi:10.1128/MMBR.00043-12.

Rühs, P. *et al*. (2013) ‘In-situ Quantification of the Interfacial Rheological Response of Bacterial Biofilms to Environmental Stimuli’, *PLoS ONE*, 8(11), e78524. doi:org/10.1371/journal.pone.0078524.

Rumbaugh, K. *et al*. (1999) ‘Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections’, Infection and Immunity, 67(11), pp.5854-5862. doi:10.1128/IAI.67.11.5854-5862.1999.

Rumble, H. and Gange, A. (2017) ‘Microbial inoculants as a soil remediation tool for extensive green roods’, *Ecological Engineering*, 102, pp.188-198. doi:org/10.1016/j.ecoleng.2017.01.025.

Russell, J. and Dombrowski, D. (1980) ‘Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture’, *Applied and Environmental Microbiology*, 39(3), pp.604-610.

Saha, R. *et al*. (2013) ‘Microbial siderophores: a mini review’, *Journal of Basic Microbiology’*, 53(4), pp.303-317. doi:org/10.1002/jobm.201100552.

Saichana, N. *et al.* (2015) ‘Acetic acid bacteria: A group of bacteria with versatile biotechnological applications’, *Biotechnology Advances*, 33(6) part 2, pp.1260-1271. doi:org/10.1016/j.biotechadv.2014.12.001.

Sambrook, J., Fritsch, F. and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbour Laboratory Press.

Sanchez-Clemente, R*. et al.* (2018) ‘Study of pH Changes in Media during Bacterial Growth of Several Environmental Strains’, *Proceedings*, 2(10). doi:org/10.3390/proceedings2201297.

Saxena, K. *et al.* (1994) ‘Characterization of genes in the cellulose-synthesizing operon (acs operon) of Acetobacter xylinum: implications for cellulose crystallization,’ *Journal of Bacteriology,* 176(18), pp.5735-5752. doi:10.1128/jb.176.18.5735-5752.1994.

Scales, B. *et al*. (2014) ‘Microbiology, Genomics, and Clinical Significance of the *Pseudomonas fluorescens* Species Complex, un Unappreciated Colonizer of Humans’, *Clinical Microbiology Reviews*, 27(4), pp. 927-948. doi:10.1128/CMR.00044-14.

Schluter, D. (2000) ‘The Ecology of Adaptive Radiation’ Oxford: Oxford University Press.

Seneviratne, G. *et al*. (2011) ‘Developed microbial biofilms can restore deteriorated conventional agricultural soils’, *Soil Biology and Biochemistry*, 43(5), pp.1059-1062. doi:org/10.1016/j.soilbio.2011.01.026.

Shahid, I., Malik, K. and Mehnaz, S. (2018) ‘A decade of understanding secondary metabolism in *Pseudomonas* spp. for sustainable agriculture and pharmaceutical applications’, *Environmental Sustainability*, 1, pp.3-17. doi:org/10.1007/s42398-018-0006-2.

Shannon, C. (1948) ‘A Mathematical Theory of Communication’, *Bell System Technical Journal*, 27, pp.379-423.

Shapiro, J. and Dworkin, M. (1997) *Bacteria as multicellular organisms*. New York: Oxford University Press, Inc.

Sheng, G., Yu, H. and Li, X. (2010) ‘Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review’, *Biotechnology Advances*, 28(6), pp.882-894. doi:org/10.1016/j.biotechadv.2010.08.001.

Shi, X. and Zhu, X. (2009) ‘Biofilm formation and food safety in food industries’, *Trends in Food science and Technology,* 20(9), pp.407-413. doi:org/10.1016/j.tifs.2009.01.054.

Shitashiro, M. *et al.* (2003) ‘Evaluation of bacterial aerotaxis for its potential use in detecting the toxicity of chemicals to microorganisms’, *Journal of biotechnology*, 101(1), pp.11-18. doi:10.1016/S0168-1656(02)00285-7.

Shridhar, S. and Dhanashree, B. (2019) ‘Antibiotic susceptibility pattern and biofilm formation in clinical isolates of *Enterococcus* spp.’, *Interdisciplinary Perspectives on Infectious Diseases,* ID 7854968. doi:org/10.1155/2019/7854968.

Siebring, J. *et al*. (2014) ‘Repeated triggering of sporulation in *Bacillus subtilis* selects against a protein that affects the timing of cell division’, *The ISME Journal*, 8, pp.77-87. doi:org/10.1038/ismej.2013.128.

Silby, M. (2011) ‘*Pseudomonas genomes*: diverse and Adaptable’, *FEMS Microbiology Reviews*. 35(4) 2001, pp.652-680. doi:org/10.1111/j.1574-6976.2011.00269.x.

Silby, M. *et al*. (2009) ‘Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*’, *Genome Biology*, 10(R51). doi:10.1186/gb-2009-10-5-r51.

Simpson, E. (1949) ‘Measurement of diversity’, *Nature*, 163, pp.688. doi:org/10.1038/163688a0.

Singh, R., Paul, D. and Jain, R. (2006) ‘Biofilms: implications in bioremediation’, *Trends in Microbiology*, 14(9), pp.389-397. doi:org/10.1016/j.tim.2006.07.001.

Slatkin M. (1974) ‘Hedging one's evolutionary bets’, *Nature*, 250, pp.704-705. doi:org/10.1038/250704b0.

Smith, V. (2007) ‘Microbial diversity-productivity relationships in aquatic ecosystems’, *FEMS Microbiology Ecology*, 62(2), pp.181-186. doi:org/10.1111/j.1574-6941.2007.00381.x.

Solano, C. *et al*. (2002) ‘Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose’, *Molecular Microbiology*, 43, pp.793-808. doi:org/10.1046/j.1365-2958.2002.02802.x.

Song, H. *et al*. (2014) ‘Synthetic microbial consortia: from systematic analysis to construction and applications’, *Chemical Society Reviews*, 6954, pp.6954-6981. doi:org/10.1039/C4CS00114A.

Sorokina, V. (1938) ‘Exchange of substances between slime and water, as influenced by the formation of a bacterial film on the surface of the slime’ [In Russian] *Microbiology*,7, pp.579-591.

Sousa, A. *et al*. (2013) ‘Improvements on colony morphology identification towards bacterial profiling’, *Journal of microbiological methods*, 95, pp.327-335. doi:org/10.1016/j.mimet.2013.09.020.

Spellerberg, I. and Fedor, P. (2003) ‘A tribute to Claude Shannon (1916-2001) and a plea for more rigorous use of species richness, species diversity and the ‘Shannon-Wiender’ index’, *Global Ecology and Biogeography*, 12(3), pp.177-179. doi:org/10.1046/j.1466-822X.2003.00015.x.

Spiers, A. (2007) ‘Wrinkly-spreader fitness in the two-dimensional agar plate microcosm: maladaptation, compensation and ecological success,’ *PLoS One*, 2(1), doi:org/10.1371/journal.pone.0000740.

Spiers, A. (2014) 'A Mechanistic Explanation Linked in Adaptive Mutation, Niche Change, and Fitness Advantage for the Wrinkly Spreader', *International Journal of Evolutionary Biology*, 675432. doi:10.1155/2014/675432.

Spiers, A. and Rainey, P. (2005) ‘The *Pseudomonas fluorescens* SBW25 wrinkly spreader biofilm requires attachment factor, cellulose fibre and LPS interactions to maintain strength and integrity,’ *Microbiology*, 151(9), pp.2829-2839. doi:10.1099/mic.0.27984-0.

Spiers, A. *et al*. (2003) 'Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose' *Molecular Microbiology*, 50(1), pp.15-27. doi:org/10.1046/j.1365-2958.2003.03670.x.

Spiers, A. *et al*. (2006) ‘A Survey of A-L Biofilm Formation and Cellulose Expression Amongst Soil and Plant-Associated *Pseudomonas* Isolates’, in *Microbial Ecology of Aerial Plant Surfaces.* P.T.N. Spencer-Phillips, pp.121-133.

Stainer, R. (1951) ‘The Life-Work of a Founder of Bacteriology’ *The Quarterly Review of Biology*, 26(1), pp.3-37. doi:org/10.1086/397881.

Stana-Kleinschek, K. *et al.* (2002) ‘Determination of the adsorption character of cellulose fibres using surface tension and surface charge’, *Materials Research Innovations*, 6, pp.13-18. doi:10.1007/s10019-002-0168-4.

Standar, K. *et al*. (2010) ‘Setup of an *In Vitro* Test System for Basic Studies on Biofilm Behaviour of Mixed-Species Cultures with Dental and Periodontal Pathogens’, *PLoS ONE*, 5, pp.131-135. Doi:org/10.1371/journal.pone.0013135.

Stanier, R., Palleroni, N. and Doudoroff, M. (1966) ‘The aerobic pseudomonads: a taxonomic study’, *Microbiology,* 43(2), pp.159–271.

Stebbins, C. (1950) *Variation and Evolution in Plants*. New York: Columbia University Press.

Steenackers, H. *et al*. (2016) ‘Experimental evolution in biofilm populations’, *FEMS Microbiology Reviews,* 40(3), pp.373-397. doi:org/10.1093/femsre/fuw002.

Stewart, P. and Franklin, M. (2008) ‘Physiological heterogeneity in biofilms’, *Nature*, 6, pp.199-210. doi:org/10.1038/nrmicro1838.

Stilwell *et al.* (2020) ‘Resource heterogeneity and the evolution of public cooperation’, *Evolution letters*, 4(2), pp.155-163. doi:org/10.1002/evl3.158.

Stirling, P. (2002) *Ecology – Theories and Application*. 4th edn. Price Hall, USA publishing.

Stoodley, P. *et al*. (1997) ‘Consensus model of biofilm structure’, in Wimpenny, J*. et al.* (ed.), *Biofilms: community interactions and controls*. Cardiff, U.K.: BioLine. pp.1-9.

Stoodley, P., Hall-Stoodley, L. and Lappin-Scott, H. (2000) 'Detachment, surface migration and other dynamic behaviour in bacterial biofilms revealed by digital time-lapse imaging' *Methods in Enzymology*, 337, pp.306-319. doi.org/10.1016/S0076-6879(01)37023-4.

Stubbendieck, R., Vargas-Bautista, C. and Straight, P. (2016) 'Bacterial Communities: Interactions to Scale', *Frontiers in Microbiology*, 8(7), doi:10.3389/fmicb.2016.01234.

Sutherland, I. (2001) ‘Biofilm exopolysaccharides: a strong and sticky framework. Microbiology’, *Microbiology*, 147, pp.3-9.

Suzuki, N., Yoshida, A. and Nakano, Y. (2005) ‘Quantitative analysis of multi-species Oral biofilms by TaqMan Real-time PCR’, *Clinical Medicine and Research*, 3(3), pp.176-185. doi:10.3121/cmr.3.3.176.

Svensson, E. and Berger, D. (2019) ‘The role of Mutation Bias in Adaptive Evolution’, *Trends in Ecology and Evolution*, 34(5) pp.422-434. doi:org/10.1016/j.tree.2019.01.015.

Szurmant, H and Ordal, G. (2004) ‘Diversity in Chemotaxis Mechanisms among the Bacteria and Archae’, *Microbiology and Molecular biology reviews*, 68(2) pp.301-319. doi:10.1128/MMBR.68.2.301–319.2004.

Tait, K. and Sutherland, I. (2002) ‘Antagonistic interactions amongst bacteriocin-producing enteric bacteria in dual species biofilms’, *Journal of Applied Microbiology*, 93, pp.345-352. doi.org/10.1046/j.1365-2672.2002.01692.x.

Takenaka, S., Ohsumi, T. and Noiri, Y. (2019) ‘Evidence-based strategy for dental biofilms: current evidence of mouthwashes on dental biofilm and gingivitis’, *Japanese Dental Science Review,* 55 (1), pp.33-40. doi.org/10.1016/j.jdsr.2018.07.001.

Takeuchi, K. *et al*. (2000) ‘Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella typhimurium*, and *Pseudomonas fluorescens* to lettuce leaves’, *Journal of Food Protection*, 63(10), pp.1433-37. doi:org/10.4315/0362-028X-63.10.1433.

Tan, C. *et al*. (2017) ‘All together now: experimental multispecies biofilm model systems’, *Environmental Microbiology*, 19(1), pp.42-53. doi:org/10.1111/1462-2920.13594.

Taylor, B., Zhulin, I. and Johnson, M. (1999) 'Aerotaxis and other energy-sensing behaviour in bacteria' *Annual Review Microbiology*, 53, pp.103-28. doi:10.1146/annurev.micro.53.1.103.

Tilman, D. (2000) ‘Causes, consequences and ethics of biodiversity’, *Nature*, 405, pp.208-211. doi:org/10.1038/35012217.

Tilman, D*. et al*. (1997) ‘The Influences of Functional Diversity and Composition on Ecosystem Processes’, *Science*, 277(5330), pp.1300-1302. doi:10.1126/science.277.5330.1300.

Tombolini, R. and Jansson, J. (1998) 'Monitoring GFP-Tagged Bacterial Cells', *Bioluminescence Methods and Protocols'*, in LaRossa, R (ed.) *Methods in Molecular Microbiology.* Totowa, NJ: Humana Press. pp.285-298. doi.org/10.1385/0-89603-520-4:285.

Tomlin, K. *et al*. (2005) 'Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia* biofilms', *Applied and Environmental Microbiology*, 71(9), pp.5208-5218. doi:10.1128/AEM.71.9.5208-5218.2005.

Toté, K. (2008) 'A new colorimetric microtitre model for the detection of *Staphylococcus aureus* biofilms', Letters in Applied Microbiology, 2008, 46(2), pp.249-254. doi.org/10.1111/j.1472-765X.2007.02298.x.

Tringe, S. *et al*. (2005) 'Comparative metagenomics of microbial communities', *Science*, 308(5721), pp.554-557. doi:10.1126/science.1107851.

Tyc, O*. et al*. (2017) 'Exploring bacterial interspecific interactions for discovery of novel antimicrobial compounds', *Microbial Biotechnology,* 10(4), pp.910–925. doi:org/10.1111/1751-7915.12735.

Udall, Y. *et al*. (2015) 'The evolution of biofilm-forming Wrinkly Spreaders in static microcosms and drip-fed columns selects for subtle differences in wrinkeality and fitness'*, FEMS Microbiology Ecology*, 91(6), fiv057. doi:org/10.1093/femsec/fiv057.

Ude, S. *et al*. (2006) 'Biofilm formation and cellulose expression among diverse environmental Pseudomonas isolates', *Environmental Microbiology*, 8(11), pp.1997-2011. doi:org/10.1111/j.1462-2920.2006.01080.x.

Valderrama, W. and Cutter, C. (2013) 'An Ecological Perspective of *Listeria monocytogenes* Biofilms in Food Processing Facilities', *Critical reviews in Food Science and Nutrition*, 53(8) pp.801-817. doi:org/10.1080/10408398.2011.561378.

Van ben Bergh, B. *et al*. (2018) 'Experimental Design, Population Dynamics and Diversity in Microbial Experimental Evolution', *Microbiology and Molecular Biology Reviews*, 82(2), e000008. doi:10.1128/MMBR.00008-18.

Vasi, F., Travisano, M. and Lenski, R. (1994) 'Long-Term Experimental Evolution in *Escherichia coli*. II. Changes in Life-History Traits During Adaptation to a Seasonal Environment', *The American Naturalist*, 144(3), pp.432-456. doi:org/10.1086/285685.

Veening, J. *et al.* (2008) 'Bet-hedging and epigenetic inheritance in bacterial cell development', PNAS, 105(11), pp.4393-4398. doi:org/10.1073/pnas.0700463105.

Vellend, M. and Agrawal, A. (2010) 'Conceptual Synthesis in Community ecology'*, The Quarterly Review of Biology*, 85(2), pp.183-206.

Venail, P. *et al.* (2011) 'Diversification in temporally heterogenous environments: effect of the grain in experimental bacterial populations', *Journal of Evolutionary Biology,* 24(11), pp.2485-2495. doi:org/10.1111/j.1420-9101.2011.02376.x.

Verderosa, A., Totsika, M. and Firfull-smith, K. (2019) 'Bacterial biofilm eradication agents: A current review', *Frontiers in Chemistry*, 7. doi:10.3389/fchem.2019.00824.

Videla, H. and Herrera, L. (2005) 'Microbiologically influenced corrosion: looking to the future', *International Microbiology*, 8. pp.169-180.

Villas-Boas, S. and Bruheim, P. (2008) ‘Cold glycerol-saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells’, *Analytical biochemistry*, 370(1) pp.87-97. doi:org/10.1016/j.ab.2007.06.028.

Visick, K., Quirke, K. and McEwen, S. (2013) ‘Arabinose Induces Pellicle Formation by *Vibrio fischeri’*, Applied Environmental Microbiology, 79, pp.2069-2080. doi:10.1128/AEM.03526-12.

Vos, M. and Velicer, G. (2006) ‘Genetic Population Structure of the Soil Bacterium *Myxococcus xanthus* at the Centimeter Scale’, *Applied Environmental Microbiology*, 72, pp.3615-3625. doi:10.1128/AEM.72.5.3615-3625.2006.

Wager, H. (1911) 'On the effect of gravity upon the movements and aggregation of Euglena viridis, Ehrb., and other micro-organisms', *Philosophical Transactions of the Royal Society B*, 201, pp.333-390. doi:10.1098/rstb.1911.0007.

Wagner, V. and Iglewski, B. (2008) ‘*P. aeruginosa* Biofilms in CF infection’, *Clinical reviews in allergy and immunology,* 35(3), pp.124-134. doi:10.1007/s12016-008-8079-9.

Wang, J. *et al*. (2016) ‘Regional and global elevation patterns of microbial species richness and evenness’, *Ecography*, 40(3), pp.393-402. doi:org/10.1111/ecog.02216.

Wasi, S., Tabrez, S. and Ahmed, M. (2013) ‘Use of *Pseudomonas* spp. for the bioremediation of environmental pollutants: a review’, *Environmental monitoring and assessment*, 185, pp.8147-8155. doi:org/10.1007/s10661-013-3163-x.

Wasserman, A. 1965. ‘Absorption and Fluorescence of Water-Soluble Pigments Produced by Four Species of Pseudomonas’, *American Society for Microbiology*, 13 (2), pp.175-180.

Watnik, P. and Kolter, R. (2000) ‘Biofilm, city of microbes’, *Journal of Bacteriology*, 182, pp.2675-2679. doi:10.1128/JB.182.10.2675-2679.2000.

Weiss-Muszkat, M. *et al*. (2010) ‘Biofilm formation by and multicellular behaviour of *Escherichia coli* O55:H7, an atypical enteropathogenic strain’, *Applied Environmental Microbiology*, 76, pp.1545-1554. doi:10.1128/AEM.01395-09.

Wellborn, G. and Langerhans, R. (2015) ‘Ecological opportunity and the adaptive diversification of lineages’, *Ecology Evolution,* 5(1), pp.176-195. doi:org/10.1002/ece3.1347.

Wells, J. and Richmond, M. (1995). ‘Populations, metapopulations, and species populations: What are they and who should care?’, *Wildlife Society Bulletin*, 23(3), pp.458-462.

Wertz, S. *et al*. (2007) ‘Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance’, *Environmental Microbiology*, 9, pp.2211-2219. doi:org/10.1111/j.1462-2920.2007.01335.x.

Wessel, A. *et al.* (2014) ‘Oxygen Limitation within a Bacterial Aggregate’, *mBio*, 5, e00992-14. doi:10.1128/mBio.00992-14.

West, S. *et al*. (2006) ‘Social evolution theory for microorganisms’, *Nature reviews microbiology*, 4, pp.597-607. doi:org/10.1038/nrmicro1461.

Whitchurch, C. *et al*. (2002) 'Extracellular DNA required for bacterial biofilm formation' *Science*, 295, pp.1487.

Whitechurch, C. *et al.* (2002) ‘Extracellular DNA required for bacterial biofilm formation’, *Science*, 295, pp.1487.

Whitely, M., Lee, K. and Greenberg, E. (1999) ‘Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*’, *PNAS*, 96(24), pp.13904-13909. doi:org/10.1073/pnas.96.24.13904.

Wiehe, W. *et al*. (1996) ‘Detection of Colonization by *Pseudomonas* PsIA12 of Inoculated Roots of *Lupinus albus* and *Pisum sativum* in Greenhouse Experiments with Immunological Techniques’, *Symbiosis*, 20, pp 129-45.

Wijman, J. *et al*. (2006) 'Air-Liquid Biofilms of *Bacillus cereus*: Formation, Sporulation and Dispersion', *Applied and Environmental Microbiology*, 73(5), pp.1481-1488. doi:10.1128/AEM.01781-06.

Wimpenny, J. (2009) ‘Microbial Metropolis’, *Advances in Microbial physiology*, 56, pp.29-84. doi:org/10.1016/S0065-2911(09)05602-1.

Winogradsky, S. (1887) ‘On Sulfur Bacteria’ [In German] *Botanical Newspaper*, 45, pp.489-610.

Winogradsky, S. (1887) ‘On Sulfur Bacteria’, [In German] *Botanical Newspaper*, 45, pp.489-610.

Winsor, G. *et al*. (2011) ‘*Pseudomonas* Genome Database: improved comparative analysis and population genomes capability for *Pseudomonas* genomes’ *Nucleic Acids Research*, 39(1), pp.596-600. doi:org/10.1093/nar/gkq869.

Winstanley, O., O’Brien, S. and Brockhurst, M. (2016) ‘*Pseudomonas aeruginosa* Evolutionary Adaption and Diversification in Cystic Fibrosis Chronic Lung Infections’, *Trends in Microbiology,* 24(5), pp.327-337. doi:org/10.1016/j.tim.2016.01.008.

Wirtz, K. (2002) ‘A generic model for changes in microbial kinetic coefficients’, *Journal of Biotechnology*, 97(2), pp.147-162. doi:org/10.1016/S0168-1656(02)00064-0.

Wiser, M., Ribeck, N. and Lenski, R. (2013) ‘Long-term dynamics of adaptation in asexual populations’, *Science*, 1364, pp.1364-1367. doi.org/10.1126/science.1243357.

Wood, S. *et al*. (2009) ‘Nitrosative stress inhibits production of the virulence factor alginate in mucoid *Pseudomonas aeruginosa*’, *Free Radical Research*, 41(2), pp.208-215. doi:org/10.1080/10715760601052610.

Wotton, R. and Preston, T. (2005) ‘Surface Films: Areas of Water Bodies That Are Often Overlooked’, *Biosciences*, 55(2), pp.137-145. doi:10.1641/0006-3568(2005)055[0137:SFAOWB]2.0.CO;2.

Wyckoff, T. *et al*. (2002) ‘Static growth of mucoid pseudomonas aeruginosa selects for non-mucoid variants that have acquired flagellum-dependent motility’, *Microbiology,* 148(11), pp.3423-3430. doi:org/10.1099/00221287-148-11-3423.

Xavier, J. and Foster, K. (2007) 'Cooperation and conflict in microbial biofilms', *PNAS*, 104(3), pp.878-881. doi:org/10.1073/pnas.0607651104.

Yachi, S. and Loreau, M. (1999) ‘Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis’, *PNAS*, 96 (4), pp.1463-1468. doi:org/10.1073/pnas.96.4.1463.

Yamamoto ,K. *et al.* (2011) ‘Trade-off between oxygen and iron acquisition in bacterial cells at the air-liquid interface’, *FEMS Microbiology Ecology*, 771, pp.83-94. doi:10.1111/j.1574-6941.2011.01087.x.

Yang, S. *et al.* (2011) 'Biotransformation of selenium and arsenic in multi-species biofilm', *Environmental Chemistry*, 8(6), pp 543-55. doi:org/10.1071/EN11062.

Yang, L. *et al*. (2011) 'Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development', *Environmental Microbiology*, 13(7), pp.1705-1717. https://doi.org/10.1111/j.1462-2920.2011.02503.x.

Yoshida, S. *et al*. (2009) 'Enhanced biofilm formation and 3-chlorobenzoate degrading activity by the bacterial consortium of *Burkholderia* sp. NK8 and *Pseudomonas aeruginosa* PAO1', *Journal of Applied Microbiology*, 106, pp.790-800. doi:org/10.1111/j.1365-2672.2008.04027.x.

Yoder, J. *et al.* (2010) 'Ecological opportunity and the origin of adaptive radiations', *Journal of Evolutionary Biology,* 23, pp.1581-1596. doi:org/10.1111/j.1420-9101.2010.02029.x

Yang, T. *et al*. (2017) 'Resource availability modulates biodiversity-invasion relationship by altering competitive interactions', *Environmental Microbiology*, 19(8), pp.2984-2991. doi:org/10.1111/1462-2920.13708.

Zaman, S. *et al.* (2017) 'A Review on Antibiotic Resistance: Alarm bells are ringing', *Cureus*, 9(6), e1403. doi:10.7759/cureus.1403.

Zavarzin, G. (2006) 'Winogradsky and modern microbiology', *Microbiology,* 75(5), pp.501-511. doi:org/10.1134/S0026261706050018.

Zhao, Z. *et al.* (2015) 'Viscoelasticity of poly (ethylene glycol) solutions on supported lipid bilayers via quartz microbalance with dissipation', Macromolecules. 2015, 48(6), pp.1824-1831. doi:10.1021/acs.macromol.5b00095.

Zhao, J. (2020) 'Harnessing microbial metabolomics for industrial applications', *World journal of Microbiology and Biotechnology*, 36(1). doi:org/10.1007/s11274-019-2775-x.

ZoBell, C. and Allen, E. (1935) 'The significance of marine bacteria in the fouling of submerged surfaces', *Journal of Bacteriology*, 29, pp.239-51.

Zumft, W. and Körner, H. (1997) 'Enzyme diversity and mosaic gene organization in denitrification', *Antonie van Leeuwenhoek*, 71, pp.43-58. doi:org/10.1023/A:1000112008026.